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THE RENIN ANGIOTENSIN SYSTEM AND KIDNEY FUNCTION
A REVIEW OF CONTRIBUTIONS TO A NEW THEORY

BY

PAUL P LEYSSAC

COPENHAGEN
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*The University Institute for Experimental Medicine
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Introd ction

Ove the last decade con ide able effort has been directed tow rds la ifying the loc l physiologi l role of the enin angiotensin sy tem for the i trarenal r gul tion of kidney function in par ticul for the gul tion of lt and water xcretion glomerula filtration t (GFR) and enal blood flow (RBF)

Th que tion concerning the ontribution of th nin ystem t the egul tion f the functions i nec s ily lated intimat ly with the phenomenon ften f ed to as glomerulo-tubular bal nce" i the fact that GFR i closely in parallel with th absol t t f ove ll ab ption f alt and wate ove f irly wid nge f va i tion both unde phy i logi al and ve al p tho phy iologi l condition Si ce the p oximal eab rption amount t 70-75% f the ot l tubul abs rption of lt and w t (and of GFR) and repe tedly ha be hown to vary in pa ll l with GFR und the onditions (g Ley a 1961; Schnermann et l 1968 Rodi k t l 1969; Le ime t l 1968; Dirks t l 1965) th focus f mai i te t i thi ont t has been di ect d t the mechanism f oupling between the te f p oximal reabsorption nd f lt tion Thus h fundame t l que tion i how th p oximal absorp ive apa ty i egul ted nd djust d to th filt tion p ss nd l ve

Tw l ve oncep have been advoc t d The l ic l th ry f rm l ed by Homer Sm th (1951) and accept d by most investig t th t he t f p oximal absorption i utomati y d us ed by change i the filt d load; eve l mechanisms f w h un ti ponse h ve been proposed The othe on- p f f d t h Copenhage point f view (the d hypothe) po t l t that th proximal ab rp- re p y p i ry i bl i dependent f the l ad pe

troduction

ve the last decade con ide able ffort ha been di ected toward l ifying the loc l physi logic l r l of the eni angioten in for the intrarenal gul tion f kidney function in par icular for th regul tion f alt and wat exc tion glomerular ltr tion te (GFR) and renal blood flow (RBF)

ne que tion concerni g the contrib tion f th renin y ten t th egul tion of these functions i nec rily lated intimately with the phe omenon fte f rred to glomerulo-tubular balance"; i the fact that GFR i cl ly in par ll l with the abs lute t f ove all ab orption of lt and w t r ove f irly wide of va iation both under phy i logical and ve l patho-physi logi l conditions Since the proximal abs rption amounts t 70-75% of the total tubul reabs rption of lt and w t r (and f GFR) and repe t dly ha been hown t ry i parall l with GFR und th se onditions (g Ley 1963; Schnermann t l 1968; Rodici t l 1969; Le ine t l 1968; Dirk t l 1965) the focu f mai i t t i thi ont t has bee di cted t the mechanism f oupl ng betwee the t f p ximal reabsorption and filt tion Thus th fund ment l que tion i how th proximal ab rptive pa ity i regul ted and adjusted t the filt tion pre su and ice ve

Two lt native oncept have been advoc ted The las i al theory f rm l t d by Home Smith (1951) and ccept d by most inve tig h the ta f p oximal absorption i automati lly djus d by change i th filt ed load; seve l mech nisms f ... cu t espons h ve been proposed Th othe con p f f d t as the Copenh gen poi t f iew (o the A q ypothe i) po t lat th t the proximal absorp- ... i ... y ... p ry iabl independ t f th l ad

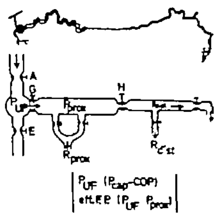
significant change in GFR are econdary to hormonally medi t d alt ation in tubul - p edominantly in proximal tubular reab orption r te (for comprehensive review of the idence l ading to thi hypoth i the eader i ref rred to Leyssac 1966)

Th vast and xpanding lit ture rel ting to th probl ms and th method logy utili ed fo thei elucid tion ha been c iti c lly reviewed by seve l investigat rs (e g O loff d Burg 1971; Wright and Gi bisch 1972; Gottschalk and La it 1973; Ea ley and Sch i 1973; d Wardene 1973; G rtz and Boylan 1973; La it r 1975) Howeve the read s of these surveys re l ft with the impre ion of conside abl uncert i ty and divergence between concepts f voured by th variou a th ; ontroversy maint ined ppa tly i part t l t by the dif f nce in per onal valuations of the po ibl influence f rrors and rtifact and henc of the ignificance f the i divid l but mutually conflicting r ult Many of the rti facts have ow been cogni d one of them n t until rece tly moti ti g re-evalu tion of th over- ll problem

In addition one of the basi hydrodynami ch ract isti of th nephron namely th r latively high flow i tance ff d by tub lar gme t di tal to th proximal tub l has lly bee neglect d i con ider tions on the dynami f glomerul lt filtr tion I my opinion thi eglect has contrib ted to much unneces ry misunderstanding and conti ued disagreement

Equally important f r th di ti ction between th v riou con cept ar l o seve l cent report on th local i t r nal actions of dogenou well ogenou angiot i whi h ont ib t t the moti tion fo e-ev l tion of the lt obt in d in th many ttempt to l ify the compl of probl ms outlined above

Th purpo f the p se t ly i is ot t give a compr hensi e iew of ll th perti t publication a ilabl F thi pu po th d i ref red to the above-me tioned views in pa ticul r to th l ant chapt i Handbook of Phy i logy (ction 8 1973 on Re l Physiology) and to the rece iew on re i l mecha i ms by D vi a d F r (1976) F th fo the ak of l ity nd comp he ion I h ve pref rred t l t mai ly those on ib ti s which I onsid be iti l nd senti l ril ton on h w y tow d ge lly acc ptabl d logi lly cn i t th ory



Classical Theory

R abs.prox. ↑(GFR)
(automatically)

1. P_{prox} constant

2. eff FP high
(30-40mmHg)

3. No hormonal regul.

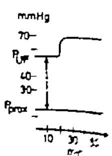


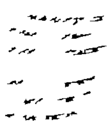
Fig. 1

The l ssi al theory The lowe ight f the f ... l pr curve ... sudden increase in the f ... (P_{UF}) has ... ff ct on the proximal lumin l pr ... d ... ance betwee the wo pr ... ur l vel i.e ... on pre ... (ff P) increas ... l ly as change in P_{UF} ... or furthe xplanat on se ...

The two lt rn tive models

The las i l theory

According to the l i al theory the r ... change pa i pa u with i ... torat ... quence f hange i GFR Th ... th prox ... t i ed ... load-d pende t ... deep t ... r th ak f impli ity the neph ... of w t pipe The re i anc f t ... middl expa ded pa t representing t ...



by mean of the taps denoted by (A) and (E) representi g th re- i tance of the ff rent and effere t rto iol s re pectively A long ide-pipe represe ting the tub le i connected with the main pipe A filt r i i sert d in the connecting link Th pres r inside th main-pipe t th l vel of the filt i e the ult afiltration press re (P_{UF}) may be ri d by change in the istance (A) and (E) i e by contr ction and dilat tion f the rteriol s P_{UF} i defined a (P_{cap} - COP); P_{ap} i th hydro- tatic pressure in th glomerul r c pilla ie and COP i th colloid osmoti press re

The filt r repr sent a cert in re istance repre ented by the tap (G) Th given v lue of thi sistance c se a given pre r drop ros th filt the magnitod of whi h i function of the re istance (G) Thi pre ure drop equal to the filtr tion pre ure minus the hydrostatic pre sur i th proximal section of the id -pipe (P_{UF} P_{prox}) determi the GFR and i r f rred to the effe tive filtration pr sure (ff F P)

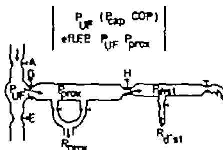
The ide-pipe (tubul) consi t of two section p oximal nd a di t l ection pa t d by a t p (H) repr enting the r i tance of th loop of He le A set of djustabl t ps connected to ach section of the long ide-pipo epre nti g tub l reab- sorption p oxi ally (R_{pro}) and di t lly ($R_{di\ t}$) r pectively It i lf-evid t th t th proximal lumin l hydro tati p is mai tained con tant if n p imary change l t us ay g an i rease n GFR autom ti ally c use parall l i creas in proxim l ab rption te such that the tap (R_{prox}) remove equ lly much more fl id from this gment is added by the change i filt tion rat Thi con tancy of proximal i tr tub la pre ur i impli it i th lassi al th ry on f it funda- me tal s umption and impli ation
Th oth r fundame t l p as umption i that th p ss re-d op on th glomerul memb ane i e the ff tive filt tion p ur i l tively high; i the rd of 10-40 mm Hg (f Pitts 1963; Pe kin and Gilmo 1973) This would imply th t th very mode t chang i p ximal int tubul p ure which might occu due t hypoth ti l p im ry change i proxim l eabsorptive pa city would t ignifi antly i fluence the magnitude of th ff c- tive filt tion p (GFR) Such p imary change i tubul reab rption if h y occu at ll then could only be r po ibl f r hang i th p ur d op (ff F P) of few pe t The f re th f filt ion would lmo t tirely be d t

ined by th magnitude of and change in the oitr filtr tion
ure (P_{UF}) i by glomerula vascul r factors

rc us f the utomati (phy io lly det rmined) par ll l adjust
nt f the proximal r absorptive apacity any h rmonal regulation
f fluid r ab orption r te by this s gment would of cours ppear
aningl acco ding to thi theory

with beli ved that th mechani m r sponsible f r the assumed auto-
tic adjustme t of the rate of p oximal r absorption was the
re tion by ctive odium t ansport at maximum capacity of a te-
imiting concentr tion diff re ce acros the proximal pithelium
We son An low and Smith 1948; Smith 1951) Thereby any incre-
t i th d livery of fluid into the tubul by filt ation would
nd t abolish the conce t tion diff rence so that the absol te
f t ansf would tend to inc as pari pa su with the fil
ration r t nd vice versa when the GFR was reduc d Howeve
t mi ropunctur tudie though first ugg ted by th classi
l tudy f Walk r Bott Olive nd McDowell (1941) tabli hed
yond any doubt that uch a t limiting concentration diff renc
oe not obtain in thi tubul segment of the nephron und normal
ondition (La ite Gottsch lk and Mylle 1961; Windhage and
iebisch 1961; Ull ich t al 1963) Furthermore the theory was
iously que tioned by re ults obt in d i tudi on dil tion
i (Wo) 1954) Supported by sults obt ined by Sel
urt i todi on pr diur i (Selkurt Hall and Spe cer
949 Selkurt 1951) Wojeson i terpreted hi d ta as vidence
bei indi ec gge ti g th t th te of reabsorption did t
h ge tomat lly i pon to hange in the load on the
ont ry he delay of abou 10 15 mi t bef re any
hange th f absorption and glomerul filt tion
and RBF) could be det t d

The lt rn tive hypothesi
on inced bou the lid y f hi co ected l ance dat
) se d h f ll log l consequenc f the observ tion
h m l ion f the t of abs rption doe t change
ma l y di ct function f the filt d load t f l
i w th p on f onst ncy f the p oximal t ubular
p te i l d onseque ly t i the te f b
p wh h d ro th f filt tion the th
w



Alternative

Reabs.prox. load-independ. (Tm)

GFR ↓(reabs)

↓P prox variable (≠P UF)

2 effLEP low (≈10-15mmHg)

1 Reabs.prox. hormonal regul.

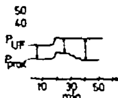


Fig. 2

Th l rna we hypothe is Th lowe gh f h f gure shows hec et l p ssr curve A udden small incre i he fi on pr ur (P F) ac ompanied i i lly by qual se prox mal lum l pre ur (P pro) The d ance b twee he we pre ur level i the ffective filt ion pre (E F P rem i unchanged i it lly Af 10 mins P prox gre down q due in reased ab rption whil P UF i re ised l d Th secondary change i P prox i the ause f t del yed re se ff F P F f rthe plan ion se

The logi f thi concl ion i very imple Let con ide by way of xampl cute increme t in the filt tion pres (P UF) Thi ch ge will t nd to incr ase th r t f filtration However if fir tly th f p oximal r ab ption doe not change automati lly i respon to the change in P UF in other wo d if no mo fl id i moved from this segme t by the tap (R pro) (f fig 2); d econdly if the t p (H) at the end of thi egment i lmo l ed bec us f th l ge re istance to flow in th loop f He l and l t r egme t th it follows th t th p o imal i tr lumi l pre will incr se cl s ly in par ll l with th filt tion p s M mor fl id will be filt red th th t requ i d t l the i t stub l r p re and to in

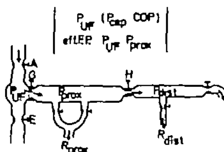
t in the p ssure t thi highe level The ffective filtration p ur will not chang to any measurabl degree not initi lly at l ast N t until the p oximal reabsorptive c pacit has in- reased and more fluid i removed from this tubular segment will the luminal pre re f ll down ag in In thi case it i the change i th rate of reabs rption which det rmines th change in th pre re drop acro the glomerular membrane i in th ffective filtr tion p ur (GFR)

It i vide t th t implied i this concept are three fundament l f ture 1) firstly the proximal intr tubul r pressure is not constant but va iabl ; varying p imarily in par ll l with the filtr tion pres re (P_{UT}) 2) Secondly the mode t change in proximal luminal p ur which an be caused by alte tion i th t f absorption must be fficiently l ge to account f the relatively l rge range of vari tion in GFR which actually can be observed Thus thi lt rn tive t the clas ical theory im- pli p requi ite that th ff ctive filt tion pre ure must be considerably lowe than pre iou ly beli ved - i e about the are value as the proximal lumin l pre (about 10-15 mm Hg i the t) lowe 3) Thirdly it i known that the rates of reab orption and filtration actually do change in pa ll l within 10 15 mi ut in re pon to induced change i P_{UT} Al o RBF change roughly i par ll l with GFR und the onditions Con- eque tly some fact r( ) au i g thi secondary nd delayed change i reab rptive c pacity must i t

The unique truct re of the nephron with it ascending limb of Henl making close ontact with both the ff t and ff re t art i l f it ffili ted glomerul (Faarup 1965; Ba j 1970) i worthy of tt tion Thi f tur which i a ch ac- t i ti of ll nephron throughout th mammalian ki gdom ugge t the i t nce f feedb k loop; and th region of thi tubulo- rul cont ct obviou ly i t t gi key-point f the recog- i ion f y co tomati dj tment f th pro im l ab orptive pacity iabl filt tion pre ur At this very egion j he ju glomerul app t (JGA) th nzyme reni i synthe- ti ed nd rel sed

A worki g hypot i

It the f re t ept og an lt rnative to the l ic l th ry f rm l d t t the lidity of worki g hypothe i i whi h th re i gi t i sy tem play d l rôl (Leyssac 1966) The ence f thi hypoth i w th f llowing (cf fig 3)



Alternative

Reabs.prox.load-independ. (Tm)

GFR ↓(reabs)

1.P_{prox} variable (↓P_{UF})

2.effLEP low (≈10 15mmHg)

3.Reabs.prox.hormonal regul.

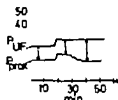


Fig. 2

Th lternat ve hypothesi Th lowe lph f the figure shows he ti l pre curve A sudden small incre se in the fil on pre ($_{UF}$) accompanied initi lly by an equal in- ase i proximal lumi l pre (P_{prox}) The di ance between he wo pres re l vel i the ffective fil ion pre re (ff F P) remains unchanged initi lly Af 10 minu es P_{prox} goe down ag i due i cre ed reabsorption whil P_{UF} i main ined l d Th secondary change i P_{prox} i the ase f h del yed mo se ff F P F furthe xplan tion see he t xt

The logi of thi conclu ion i ry simpl Let consider by w y of mple acut increme t in the filtr tion press re (P_{UF}) Thi change will tend to incre th r t of filt ation However if fir tly th t f p oximal r ab orption doe not change a tomati lly i spon to the cha g in P_{UF} in other words if no mor fl id i moved from thi gment by the t p ($R_{p\ ox}$) (f fig 2); and econdly if th t p (H) t the nd of thi s gm t i lno l d be f th l ge resi tance to flow i th loop of He l d lat gme t then it follows that the p o imal i t lumi l p will incre clos ly i p r ll l with th filt ti p r No mor fluid will be filt r d than th t r quired l t th i t t b l p re and to mai

tal the p ssure t thi higher level The effective filtration p s re will not change to any me surabl degree n t initially t l ast Not until th p oximal reab rptive c pacity has in cre sed and mo fl id i removed from thi tubul segment will the luminal pre sur f ll down g in In this ase it is the change i the rate of reabsorption which det rmine the change in the pre sure drop acros the glomerular memb ane i in the ffective filt tion pre ur (GFR)

It i vide t that implied i thi concept re three fundamental f ture 1) firstly th proximal i t tubul pressure is not constant but va iabl ; varying prima ily in pa ll l with the filt tion p re (P_{UF}) 2) Secondly the mode t change in proximal luminal pr sur which an be used by ltar tions in the r t of ab rption mu t be suffi i tly l rge to account f the rel tively l rge ange of v ri tion i GFR which actually can be observed Th thi lt rnative t the las i l theory implies as a prerequi it that the ff ctive filtr tion p es re must be considerably lowe than pr viou ly beli ved i about the are value as the p oximal lumi l pres re (about 10-15 mm Hg in the t) or lowe 3) Thirdly it is known that the t of reabs rption and filt tion actually do change in p rall l within 10-15 mi t i re ponse to induced changes in P_{UF} Als RBF change roughly in par ll l with GFR under th conditions Consequently one factor( ) c using thi ond ry and delayed change i reabs rptive pa ity mu t i t

Th unique truct re of th nephron with its scending limb f Henl making lose ontact with both the aff ent and ff t art l l of it ffili ted glomerul (P rup 1965; B j 1970) i wo thy f ttention Thi f ture which i a cha act i ti of ll nephron throughout the mammali kingdom sugge t the i tence f feedback loop; nd the gion f thi tubulovascul contact ob iou ly i t t gi k y-poi t f th recognition f y on tons i dj tment of th p oxi l ab ptive pacity t iabl filt tion pre At thi ry egion i the juxt glomerul ppa t (JGA) th yme re i i ynthe ed and rel ed

A workin hypoth i
I w h f t pting an lt rn ive t tl l l th ry f rr l t nd t he lid y f wo king hypothe i whi h he re i gl t l my pl yed t l) (Ley & 1966) Th ence f thi hypoth i w th f llow g (f f g 3)

Reni rele se is ugme t d unde conditions i which th filtration pre sure tend to decline (cf also recent view by Davis and Freeman 1976) A drop i P_{UF} is immediately accompa ied by an equally large drop in the proximal int tubul r pr s ure (P_{pro}); consequently the rat of delivery of tubular fluid into the dis t l tubul d cre s and th distal intr luminal pres ur goes down As a result urine flow decrea s

Th reduced delivery of fluid from the scending limb of He l stimulat nin r l as omehow a a feedback re ponse pos ibly vi th mac l den ; d angiot n in II i subs que tly formed Angiotensi II might h ve two ff ct : 1) Firstly th well known va ocon tri tory eff ct If thi wer an ffect p edominantly on the post-glomerular rt ri l a lik ly possibility ince the eff rent ve sel would be the first smooth mu l cont ining rt riole met by the ndogenous angi t nsin II liberat d i t a re ally - th post-glomerul r vascul resistance wo ld i cre the eby count racting th primary drop i P_{UF} The p oxim l i tr tubula pr su would therefore increa somewh t but not n ce sa ily compl tely back to the origi l v lue In addition th eff re t v oconstri tion modi t d by ngiot n in would contrib t to the r du tion i enal blood flow (RBF)

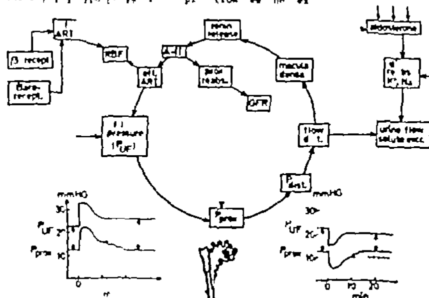


Fig. 1

The wor l g ypo he is f pl tion ee he ex

2) Secondly a hypotheti l inhibitory ff ct of angi ten in on the proximal reabsorptive c pacity could be post l ted Inhibition of proximal reabsorption r t would further l v t th proximal luminal p sure whi h then would return right back to the control l vel It i pparent th t thi l tt r part of the int atubul pres ure incre se i the major det rminant of th resulting reduction in the ffective filtration pre sure and hence in GFR. Furth r in thi f hion the two angioten in effects would influence th i tr tubul pre sure in the same direction and assi t ach oth r in maint i ing th stability of the pre sure

As re ult f uch feedback mechani m RBF proximal reabsorption r t and GFR would v ry in par ll l as obs rved; and proxi mal intr tub lar pre sure and th reby the delivery of fluid to more di t l tubular segment would be maint ined in stable t t

According to thi working hypothe i compl t corre pondence and agreement with the las ic l theory i r ached in the t ady t tes. Even load-dependency of pro imal reabsorptive c pacity is an e enti l ch act ri ti in both concepts being a direct and utomati d pendency according to th on but being an indirect dependency according t the the concept Thus the pre ent working hypoth i y be con idered unifyi g hypothe i Only the mechanism by which change i the system accompli hed diff entirely f om the la i l theory; there i fundame tal diff rence in th equence of ve t

In h f llowi g sections th iou tt mpt t t t the new worki g hypo he i ve su the l i l theory re reviewed The he rt f the probl m w o obt in direct id ce tabli hing wh the he absol t t of proximal reabs rption ri uto-ma i lly di ct fun ion f the filt red l ad; r wh th i i p i a ily ind pend f GFR Th ch enti l que tion were wh he not angioten i II may inhibi he pro i l reb rp ve per l y and add ion act p f re i lly on h ff l

Evide ce ob ned w th he oc l ion ime tec ique

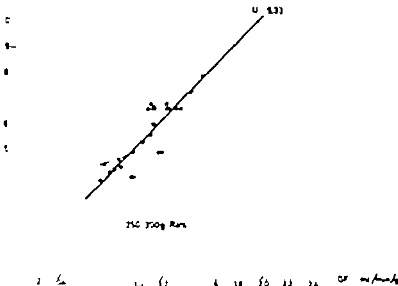
A rel ively impl hnique sui abl f g some f the l que on p ovided with he i roduc on f h oc l on me me od f me ng ab l f proximal net f f l nd w h (Ley ac 1963) Whil he t f he dney ob rved with rec copi i ro ope tle ly ompl ly larped above l rt i As f he ab p l up on f h l pply th

filtration proce tops instantaneously whilst th proximal reabsorption conti ues until th lumi a re rpti d and totally occluded Th time int rval from rtic clamping until th proxi mal lumin ha e di ppeared i r corded and den ted by th occl sion time (OT) Th t rm occlu ion time was profe ed to col l pe time in o der t di tinguish th g adual luminal occl sion du to r ab rption from the sudden coll pse of the p oximal lumen re lting g from applying too much uction to an in rt d collecti g mi ropipett

If reabs rpti c pacity i onsta t duri g th lumin l occlusion the

$$C \quad \frac{}{OT}$$

in whi h C represent th p oximal reabsorpti pacity and th lumi al adi s F r any given lumi l di the OT will be mor r pid the l rg th reab orptive r t Conver ely the more the tubul s r distended th longer the OT for any given v lue of C Thus knowi g the lumi l radiu th ab ol t r t of p oximal reabs rption can be c l lated from th mea ured OT



Result equal to thos given in fig 4 obtained within the range of pontaneous v ri tions in inulin cl rance from 0 7 to 2 0 ml min [1] per g of kidney weight (k w) in r ts demonst ted th t the reciproc l f th OT vari d line rly and in direct proportion to the GFR me sured just bef re th aort w uddenly lamped (Leyssac 1963) I ddition it was documented th t unde the conditions the proximal lumin l radius was on an aver ge 13 14 μm in ection of qui k frozen on-diureti rat kidneys independe t of th in lin l rance t lue above 0 7 ml min [1] g k w [1] These result demonstrated the ph nome on of glomerulo-tubul r balance But the crux and signifi ance of thi xperiment was th t lmo t identi l v lue of ab lut proximal reabsorption rat were found whe lcul ted both from the i ulin l ance and the proximal fraction l reab rption th latt r known from fr flow mi ropuncture dat and from the me ur d lumin l radius and the OT Thi los greement seemed to justify the conclusion th t pro imal tub l r reabs rption continue throughout the oc l ion ime t an unchanged t ven though the filtr tion r t drops ro h cl r and direct pl ce of ide ce indic ting th the of reabs rption doe not hange utomati ly in re pon to l tion in GFR h d th apparently been obt ined

Furth r it has bee documen ed th t pro imal oc l ion time can be qu antit ively rel ted o the othe paramet rs depend t on proxi mal ub l reabs rption t th t re mea ured in free flow such tr nsit me (f th dye front f bolu of filt red li amine gree) and proximal f actional reab rp ion (and he ce h tub lar fl d pl sma in li io $(TF/P)_{IN}$) Th unique rel tion hip be ween OT an it time (TT) nd TF_{IN}/P_{IN} h lso bee derived (B) and Ley sac 1969)

$$\frac{TT}{OT} \quad \ln\left(\frac{TF}{P}\right)_{IN}$$

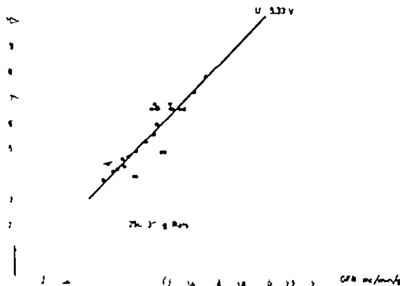
W h p ope rect on f node t (12%) re rograde i flow f fl id from he p rec f he p imal tubule duri g the p oxi al oc l ion ph ome on l quantit ive gre me be ween al l ed and di ec ly me d lue f in lin l ance (Cl_{IN}) TF P i l t TT rr cted OT and lumi l adi has be n f und ove he ge f pon aneou i tion (Bo) n and Ley 19 l l od an qu lly los q antit tive r me be we It ob ined by he inve ig rs w l de diff re cond ior of se tr i and t el me xpan on (de i f) h rong id ce d T me od h be ol d nd th co l ion

filtration proc top in t taneously whilst th proximal reab orption continue until th lumina re empti d and totally occluded The time i t rv l f om aortic clamping until the pro i mal lumina ha e di ppeared i r corded and den ted by th occlu- ion time (OT) Th t rm occlusion time was pref ed to col l ps time in ord r to di tingui h th g adu l lumin l occlusion du to eab orption from th ddo coll pse of th p oximal lumen re lti g e g from applying too much uction to n inserted oll cting micropipett

If abs rptive capacity is con tant d ing th luminal occl sion the

$$C = \frac{r}{OT}$$

in which C repre ts the proximal reabsorptive c p ity and the lumi l dius Fo any given lumi l r dius the OT will be more rapid the l rg the ab rptive t Conversely the mo the tubul s di tended th longe the OT f r y given v lue of C Th knowing the lumin l radi th absolut te of pro imal r ab rption an be c lcul ted f om the me red OT



Res lts equ l to those given in fig 4 obt ined withi the ange of po taneous v ri tion in i ulin l arance from 0 7 to 2 0 ml min [1] pe g of kidney weight (k w) in r ts demon tr ted that the eciprocal f th OT v ried linearly and in direct proportion to the GFR me sured just bef re the ao ta w udde ly clamped (Leyssac 1963) In addition it w s documented that unde the e condition the proximal lumi l radi wa on an ve age 13 14 µm in ction of qui k frozen non-diureti rat kidneys independent of th in lin clearance t alue above 0 7 ml min [1] g k w [1] These results demonst ted the phenomenon of glomerulo-tub lar balance But the crux and signifi an of thi xperiment was that lmo t identi l value of abs l te proximal absorption t were found wh n calcul ted both from th inuli cl rance and the proximal fraction l reab orption the latter known from free flow mi ropunct dat and from th measur d lumi l radi and the OT Thi los agreeme t seemed to justify the conclusion that proximal tubul reab orption continue throughout the oc l ion time at unchanged rat ve though the filtr tion r te drops t r A l r and direct pi ce f vide ce indi ating th t th t f ab orption doe not hange tomati ily in re ponse t lt tion i GFR had th pparently been obtained

F rthe it h s bee documented th t pro imal occlusion time can be quantit tively rel ted to th oth paramet rs depende t on p oxi mal tubul abs rption t that re measured in f ee flow uch nsit time (f the dye front f bol s of filt red li amine green) and proximal f tional absorption (and hence th tubul fl id t pla ma i lin ti ($^{T}T/P)_{IN}$) The unique rel tionship be wee OT t an it time (TT) and TT_{IN}/P_{IN} h lso bee derived (Boj se and Leys 1969)

$$\frac{TT}{OT} \quad 1 \; (\frac{TF}{P})_{IN}$$

W h propo ction f mode t (12%) ret ograde i flow of fl id from th pars t of the p imal tubul du ing the proximal oc l ion ph one on los qua tit tive greeme t between l cula ed and di ctly meas d lue f i li l nce (Cl_{IN}) TF P l a i TT rrect d OT d lumi al adius has bee f und ov he ti ang f pontaneou i ion (Boje and Ley sac 196) I l t tudi e equally l q antit ive j rr be we re lt ob i d by th inve t g tor w TP al nd d ff ondition (use the i and e t cell l l me pansion (ide l f) Th t ong ide ce l d ng h OT ethod h been obtained and h con l i n

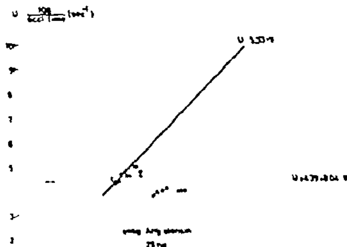


Fig. 3
Recip ocal OT ft i i jection f amglet i II t diff learance f inuli ve h pom mrows Amge f ion measur d immedi ly bef the admi i t ion f mg i The reg ion line U 5 31 Y i th obt ined can rol mot q ve amglet i (Ley ac 1965)

d awn by Gottsch lk and Lassit (1973) th t the OT method wh ppli d c rr ctly i a imple and accu t mea urement p oviding qu tit tively correct data on ab ol t t of proximal ab orption seems j tifi d

It f llows that the t tement that proximal r ab orptive rat i a ptima y riabl ind pe de t of the GFR per s must be equally co t

Utili i g th ne methodology it w hown that ogeno glo t a ll inj cted i tr ualy just ft the uri and blood mpli g f ontrol l r nce me rement p olong d the OT w re d immedi t ly (i 3 c) ft r th pa ge f glo t h g th kidney Th re lt (given in fig 5) were i t rp t d di id c that gl t in depr d the p t I ab rp ive pacity from any high l vel down t ni i m lue q I th lowest l cord d within the pr t e j i ti (Ley 1964 1965) It hould be j 4 d t i hibitor tubul r ffect of glot al c ld e i l d ly t th high t pont ne us con r l t of r ab t i f l ti (cf lso l t r) Th inhibitory

ffect of angiotensin wa dose-dependent while equipre sor dos of noradrenaline had no immedi te effect on the OT (Leyssac 1965)

The problem of proximal luminal diamet r vari tions

Provoked apparently i p rt t l ast by the evid nce obt ined with the OT technique seve l groups f inve tigators ngaged themselves in t ting th result with more sophisticat d mi ropunctur methodology The inability i th a li r report to reproduc the Copenhagen re ults re ted some confusion and gre t difficulties in xplaining the dis repancies Almost ll other groups consi t ntly found that th proximal lumin l radius va ied linearly with th ate of filt ation such th t the luminal volume pe unit of tubul r length ( ) in re ed in direct proportion t GFR. In addition r lts obt ined with the plit-oil drop method i trod ced by Ge t (1963) showed th t the reab orptive half time ($t_{\frac{1}{2}}$) w onstant and independent of lt r tions i y teni rt rial pressure and GFR (Gertz t l 1965)

According to Ge t

$$\frac{0.693}{t_{\frac{1}{2}}} \quad \frac{C}{r^2}$$

i which C gai epresents the pro imal reabe rptive apacity Based on these findings Ge t p opos d that C was us lly re l ted to th luminal volume pe unit of l ngth ( ) uch th t any inc se i tubul di ten ion ulting from increme ts i GFR tomatic lly will induce par ll l change in the t of ab orption Thi hypothe i wa pidly accepted by most i ve tig t in the fi ld; f seve al y it w considered firmly establi hed by upporti g e idence from th split-oil drop d t an it time xpe ime t (Recto t l 1966; Brunne t l 1966 Rect r t al 1967; Schnormann t l 1968) Apparently the arli eport (Leys ac 1963) th t equally large i tions i prox mal reab orption t ould be obs rved with out any i tion i the luminal diamet w di g rded ven t ough i would ppe trong gume t g i t th concept that d ane ch nge physi lly use p imal reab rption te to ha ge om ti lly The que tion w th f r inve igated h eproved chn que nd i bli d-expe ime t (B ino Gott ch l and Ley a. 1968) l vivo mi ph tog ph we made of fac cn l t f kidney du ing th collection ye od f l anc me re t t l rg nge f teady t t f l m l g from pe i l on tri tion of the renal y m u g pon ane ly Confirmi g th crigin l ob

v tion o chang in p oximal tubul r diamet r could be d tect d ov r the range of pontaneous vari tions; at lower value of GFR th adius act ally tended to increase Not until l t r wa it gene ally recogni ed that the esults obtained from split-oil drop experiments were due t artifacts (f c iti l review of th method by Orl ff nd Burg 1971 and b Gott chalk and La it r 1973) which can readily be xpl ined (cf Ley c and Boj se 1966)

S riously ch llenging th occlusion time method Wahl t l (1967) in Th u labor t ry r ported th t th c urse of reduction in proximal tubul radius duri g the occl ion ph cmenon me ured i photomi ogr ph taken t l ec i terval w s linear func- tion of time Even though t me emert of the adiu (plot ted on the ordi t) could be obt ined only du ing th first 6 eo th lope of the li e l ti g adi s to time w s uffi i tly xa t f r th c l ulation of the i itial r t f r abs rption and f th declin i radius E tr pol tion of th lines tarti g f om a radi (o) of about 10 inte cept d th ab is t a pol t qual to the me sured OT If r ab orption r t h d mai d constant d ring th l minal oc lu ion curved line should be obt in d Th r fore the e d t were int rp ted vidence that th t f r ab orption conti uou ly and tcmati lly decre e pari r u with th decre i di gg t d by Ge t However th p oblem i volved i me ri g the pro imal lumi l di i ph tomicrog aph had ot bee conside d Me urements f th proxi l dius g ne lly did t i lude the ref actil l (on) p sent in ph tog aph t th ut margin f the tub l lume givi g a radiu f about 10 um lso found by oth (Bai t l 1968) Th probl n w olved in g t F l t by t dying ph tograph mad of Pl xiglas tube with unpoli hed lumi l urf ce ubmerg d into w t and xpos d with J t light thus imit ti g the condition i hich en l urfa on ol tions r photogr phed in vivo (B j and Ley- c 1969 Th ph og ph di losed th t the r f til lin i p f h l e th th igi l ph tog phs f Bain d hi oni t me ur d to i lul th fr ctil zon pro i l i f 13 14 m w found i g eement with the li me t obtai d i ection of qui k fro en kidney (Ley 1965) j l ith the lect l l radi lcul t d by H gel r i ki 1 67) Thus the adi t time o (r_o) of 13 W l l i i co rect: the the cor r ct d li 14 U i g thi co ect d lue

and the initi l lope of the line an xtrapol tion int cepts th abscis t 19-20 ec f beyond the me ured OT of 13-14 sec Howeve c lculated curve de ibing constant te of absorption given by the initial lope and tarting from radius of 14 um intercept the abscissa t v lue f 13 14 sec equal to the measured OT Thus in lity the data r ported by Wahl and hi colleague were i compatibl with Gert hypothesi whe as they lend trong upport t the occl sion time method and th pre t working hypoth i

Also ch ll ging the int rpr tation of the results obtained from oc lusion time xpe ime ts wer dat obtained in proximal tubule micrope f ion xpe imgnt *by Wiederholt and hi associ t (1967)* The res lt uggested that whil the luminal diamete increased with pe f ion te g ing from 10 to 24 nano-l per min reabsorption rat increased proportion lly ince the collect d perf ate/infused fl id i uli r tio emained unchanged A los an ly i of th i dat disclosed however th t lik ly l ak ge rtifact could xplain thei sults (f discussion by Boj en and Ley sec 1969); and l t r tudi by Morgan and Berline (1969) *ing the ame methodology were unabl to onfirm the* re ult f Wiederholt t l The rate of proximal reabsorption remained unaff cted by l rge hange in the luminal pe fusion t As poi ted out by O l ff and Burg (1971) the method f *mi roperf ion i of loe i xcluding th influence of th* glomerulus itself on net reabsorption The result obt ined by Morga d Berline therefo add additional t g idence indi ting th t the proximal ab orptive ap city is ind pe dent f h load prese ted to the lumin l ide I l t tud Steven (1974a) ived t th ame concl ion from r lt obt ined by th method f cont ll d uction during coll ction of proximal tubul fl id f th d t rmi tion of ingl nephron filtr tion t (S GFR) A 3 mm Hg reduction i proximal luminal pro re gmented SNGFR b 5 ano-l mi ^{1} g Kw ^{1} whil T P i li ti de d by 0 5 (f om 2 44 t 1 94) The absol t r t f p oxi mal ab rtion mained unchanged

Fi lly t i pe ime t was de igned by Gert d i g penh g i de t t t the validity f th occl ion me me hod nd he que ion whethe or t change in GFR lw y accomp i d by p oportion l chang in proximal l inal adius) L ine gree t an it time (TT) l pli -oil d p nd OT ould only r mai con ta t if ( ) v i d i d p rtio t on GFR Con i red that th TT would be con t t

vation o change in proximal tubular diamete could be d tected ove the ange of spontaneous variations; at lower values of GFR the radius actually tended to increa Not until late va it generally recognized that the results obtained from split-oil drop experime t were due to artifacts (f cr ti al reviews of th method by Orloff nd Burg 1971 and by Gottschalk and Lassiter 1973) which can r adily be explained (cf Leys c and Boj sen 1966)

Seriously challenging the occlusion time method Wahl et al (1967) in Thurau laboratory reported th t the cour of eduction in proximal tubular radius during th oc lusion phenomenon measured in photomi rographs taken at 1 sec interval was linear function of time Even though exact measurements of the radius (plotted on the ordinate) could be obtained only during th first 6 sec th lope of the line lating adius to time was suffi iently ma t for the cal ul tion of the nitial rat of reabsorption and f the decline in radius Extrapol tion f th lines tarting from r dius (r_o) of about 10 μm int rcepted the abscissa at a point equal to the mea ur d OT If absorption te had remained constant during the luminal occlusion curved line should be obtained Therefore these data were interpreted as evidence that the te f reab orption continuously and automati ally decre se pari passu with th decrease i radius as sugge ted by Gertz Howeve th p oblems involved in mea uring th p oximal luminal radius n photomicrog phs had not been consider d Measurements of the proximal adius gener lly d d not include the refractile line (or) present in photographs t the outer margin of th tubular lumen giving adius of about 10 μm as lso found by others (Baines t al 1968) The p oblem we lved in a gr t part t least by studying photogr phs made of a Plexiglas tube with unpolished luminal urf ce submerg d into water and exposed with inc dent light thus imit ting the conditions in which enal surface onv l tions ar ph tographed in v vo (Bojesen and Leyssac 1969) The photogr ph disclosed that the refractil lin i part of th lumen Whe th original photographs of Bain and his oci tes we re-measured to in lud the refractile one proximal adius f 13 14 μm was found in greement with the arli measurements obtained in ections f quick-frozen kidneys (Leys ac 1963) and lso with the lectri al adius alculated by Hegel Frömt and Wick (1967) Thus th radius t time zero ($_o$) f 10 μm g en by Wahl t al i incorrect; rathe the corrected adius hould be lose to 14 m U ing this correct d value

cant change in arteri l plasma oncotic pre ur (COP) g ve rise to a modest but ignificant immedi t proximal lumin l p ssu e l vation ave ging l l mm Hg which pe i ted throughout th in fusion (1 mi) Thi pr ure incr ment w ascribed to the re lting change in plasma volume Infusion of an amount f c lloid f ee solution (0 ml) whi h gave the same pl sma volume xpan- ion as timated from the change in hematocrit caused more mark d immedi t proximal lumi l pr ssur increase to an ave ge f 3 8 mm Hg and a drop in plasma COP Th diff r ce i the arly int atubul p su increme t 2 7 mm Hg lmo t exactly equal d the me sur d drop in COP of 2 6 mm Hg Sin the latt r change in COP qual the diff ence in filtr tion pre are caused by the dilution f plasma protein the r sults we di ct evi dence indic ting that th proximal luminal hydrost tic p ur i initially clos ly in pa ll l with the filtr tion pre ur (P_{UF}) in acco dance with the ltern tive wo king hypothe is In addition it w documented th t the ly intr lumi l p chang were g dually compensat d f i an undul ting manne The p ur w gui ted back to xactly the p e-infusion lue within 10 15 minut in close greement with Boj sen i terpretation of hi l a ance data (1954) and in acco dance with the data obt ined by Gottschalk and Myll (1954)

\ ry ce tly Knox (1975) ported lt obtained in dog demon t ti g enti lly the ame ph nome on Infusion f ac tyl choli i to th n l t ry acut ly ugment d RBF and markedly inc d th p oximal i t tubul p u Si gl neph on fil t tion te (SNGFR) and hence th ff ctive filt ation pr s emained un hanged whil ff re t rt i l oncoti p re de ased ignifi tly

<u>The eff ct ve filtration pr r</u>

P ious indi ct imat f glomerul apill ry hydro t ti p and permeabil ty f om i ing dat omp h ively ewed by Re ki nd G lmo (1973) l pertinent fte the d velopme f mo d ct method fo imati g the ope tive f pon ibl f glomerul lt filt tion These l tt method and the i obt ined by thei t ll tion have rece t ly be criti lly iewed by La ite (1975) The impo tance in h p sen on t f h f rmation p ovided in the tudi w b f umm ry t thi poi t i ho t p tendi g t add b l l he p ent ion give by Las it

l n ll d coun i me ing de ice n l q) f d ct co di g f hyd ost t p

ure in microvasculatur in a mutant Wi tar train of rat (th Munich train) with ve al glomerul located just underne th the capsul Brenne Troy and Da gharty (1971) first su ceeded in measuring directly the glomerular apillary p sure (P_{ap}) in ats P_{cap} veraged 45 mm Hg in hydropenic control t This value has bee confirmed repeatedly w th imilar methodology both by Brenne and hi asociat (Brann et al 1972; Robertson t l 1972; Dean t al 1973b) and by Blantz and associ te (Blantz et l 1972; Blant 1974) Ultramicro-protein det rminations on sy temic arte i l and ff rent arte iolar plasma the l tt col l cted from the tar vessels yielded ff re t ( ) and eff rent ( ) arte iolar oncotic pres ures of 20 mm Hg and 35 mm Hg respectively With a hydrostati pres ur i Bowman c psule equal t about 10-12 mm Hg as usually ecorded in early proximal convolution th net d ving f ce fo ltrafiltr tion i th ff ctive filtration pr ssure i 13 15 mm Hg at th ff ent d of the glomerula c pillari and 0 mm Hg r lightly negative t the ff nt end; hence f ltr tion equilib um i achi ved t o shortly before the end of th glomerular c pillaries Accordingly the mean ff ctive filtr tion pressu is abo t 10 mm Hg This value may be omewhat unde timated howeve The problem f correct sampling f ff rent arte i lar blood and the techni al difficultie in the ltramicro-chemical protei measurement were mphasized by Las it Furthe Allison Wilson and Gottsch lk (1975) h ve shown that d ring a micropuncture experiment th sys temi plasma prot i concentration of rat i gradually d lining in their experiments from about 5 7 g% before surgery t about 4 6 g% withi 150 min te ft r surgery corre ponding t re duction in calculated oncoti pre sure from 17 5 to 12 5 mm Hg Unpublished observ tions in our labo tory have confirmed imil albe t l ghtly l duction in directly measur d systemi on coti pre sure (COP) f llowing surgery Immediately ft anaes the ia values of 18 19 mm Hg were corded while during the mi ropunctur xpe iments COP d cli ed and verag d 17 2 ± 0 6 and 16 1 _ 0 3 mm Hg respectively n two series of xperiment (Leyssac Frederik en and Skinne 1975) If imilar value pply t Brenne ts it que tionabl wheth f lt tion equilib ium actually had been achie ved i his experiments

U ing the method of top-flow pre ure measurement (SFP) indirect estimates of glomerul apill ry pre re an be obt ined ince P_{ap} hould equal SFP plus The valid ty of correct estimat of $P_{c\ p}$ in thi fash on depends on rtain as umptions as La it

correctly mph isad; in particul r on the as umption th t the eduction in flow to th di tal tubol by the oil blockade has no ignificant hemodynamic ff cts In view of the vidence (ide inf) indi ati g a feedback mechanism i th macul d n a re ult ing in lowe v lue f SNGFR from distal c ll ctions (with intact flow to the macul de a) th f om proximal ll ctions uch in di ct measur of P_{cp} may be ubject t an artifactual ove timat t l ast under certain condition and hould be v luated with ome autio U ing thi indir t pproach Allison Lipham nd Gottschalk (1972) found a glomerula c pillary p su of 52 2 mm Hg c lc lated from a SFP of 37 5 mm Hg nd sy temi pla ma COP of 14 7 mm Hg Källskog nd as oci te (1975) in Ulfen dahl s labor tory report d imil r SFP f 38 mm Hg in t As uming a plasma protein concent ti of 6 g% equal to a COP of 20 mm Hg they lcul ted P_{p} to be 58 mm Hg However a COP f 20 mm Hg is p obably an ove timat ; from more likely lue of 16 mm Hg pplying to th nditi p v iling du ing their pe ime t P_{cp} would be 54 mm Hg i acco dance with Alli on and her oci t Käll kog t l we also able t measur the glomerul c pill ry pre di ctly in thei Sp ague Dawley t The reported v lue of 62 mm Hg i markedly highe than thos meas ed by B nne and othe in Munich Wist r rats but probably l o high than the indi ct timate f P_{p} in thei own ri f Sp ague Dawley t ggesting that speci diff ce cannot plai the di g eeme t which probably re mai an undet rmi ed techni l probl m

d

Eve though ompl t ane t f the glomerul apill ry hyd ost ti pr ur in non-diu ti t h ot been reached t p ent the bulk f idenc defi it ly how that it i much lowe than p iou ly umed A mean i f about 50 mm Hg would ppea lik ly Al y t m pl ma oncoti pre of about 16 mm Hg d ing mi opunct cond tion t i so abl value give a f lt tion f action (FF) f 0 3 the ff ent oncot pr ur will h e ise t 27 mm Hg h the me glo-merul oncoti p will be 22 23 mm Hg Th p oximal tubu l p t und these condition i about 12 mm Hg but the p Bowma pa h bee me ur d lightly high tha h me ur d i th proximal onvol tions f the e kidney Käll kog l 1975)) lue f abo t 14 mm Hg may be ason abl g g an ff ctive filt ion p f ave g 11 mm Hg f m i t l t th ual lue f he ff f ll ry accordi g he ta f the an mal

r nge of variation corresponding to the spontaneous vari tions in GFR (0 7 1 6 ml min ^{1} g kw^{-1} in hydropenic rat)

In concl ion it has been tabli hed th t the effective filt tion pres ur i low in th ord of 10 15 mm Hg demonstrating much lower flow resi tance acro the glomerula memb ane than previously suspected Thus a change in proximal luminal pre ure of only 2 mm Hg c used by primary change in the reabsorptive capacity o oth rwise ll oth factors being equal will sult in 15 20% change in the ate of filtration

Phy ical f ctor adj tment of proximal reabsorptive r te and pl sma flow dependence of GFR

Recogni ing the f ilure in demonst ating an automati and direct dependency of proximal reabsorptive capacity on th filte ed load pre ented luminally and the ucces ive rejections of the hypo- these firstly f gradient limitation seochdly of luminal flow velocity and thirdly of tubular distension (r) most i ve tig tors in the fi ld f lt tisfied for asons not di ctly pparent by the ida th t physical fact rs cluding hydro- tati pre sure (Ea ley Martino and Fri dl r 1966) and in particul the oncotic pressure (Lewy and Windh g r 1968; Wind hager Lewy and Spitze 1969; Spitze and Windhage 1970) in the pe itubular environment ignificantly contr l th rat of net proximal tran fe of lutes and wate Th concept as outlined by Windhager and adopt d by Brenne and hi group we that pri mary changes in GFR and thereby in th filt tion f action (FF) levate th ff rent oncotic pressure; thi change in Starling f rc across the peritubular apill ry wall would facilitat th net ptake from the int rstiti l pace and thereby somehow ffect (increase) the net flux of fluid across the p thelium automati ally It wa beli ved th t glomerulo-tubular balance ould be xplained by such phys l mechanism It i worth noti ing that most of the arly vidence taken as upport for thi ide was obtained with the split-oil d op method (Lewy and Wind hage 1968; Windh ge Lewy and Spit 1969; Spit and Wind- h ge 1970); and as wa lso the case with the -hypothesis whil it wa popul many investig tors found and find upporting vidence in what wo ld ppear fortuitous co-variances and re- produ ibl art facts Howeve given the premise first that chang in fl d delivery into th proximal tubul by f ltr tion (otherwise) does not influence th reabsorptive pa ity; second th t the flow esi tance f th glomerul pillary membrane i much lowe than ha ff red by the nephron segments di tal to the

proximal tubul it f llows logic lly th t any hange in filt a tion pressure (P_{UF}) is accompanied by p rall l p a ure change in the pace of Bowman h al bee demonstrated by direct measurements Thu the net driving f rce fo filtration (the ff F P) i not ignificantly altered by uch changes per se Windhager id a can the f r now priori be di regarded s logically incon ist t ince GFR is contr t to th filt tion p ure cannot inc ase ignificantly until the tubular reab- orption t ha changed (oming tubul r flow r i tance onstant under physiological conditions) Consequently hange in eff nt oncoti pr sn assumed to ause th change in re absorptive r t cannot hange bef but only ft r the change in r abs rption r te have been accompli hed Not rp i i gly an inc asing amount of evidence cont adict th idea of ignifi cant role f peritubula oncotic p es ur if any at all in the adju tments of proximal bs rption r t Mo eove the idea would requi an incredibly high ensiti ity f the reabsorptive proce to change i the oncoti p ur f th peritub la envi onme t the c pacity of the pe itubul capill i t absorb w te befo limiting duction in COP ha bee reach d not only depends on the plasma flow emphasi ed by La ite (1975) but also the at of wat t an f r from the proximal lume t the i t rstitium nd/o c pillary i ignifi ant Since th t of filtr tion and p oximal reabs rption very i pa ll l unde no t conditions the diluting ff ct of th reabs rbat on the pe i tub lar prot i concent tion would in pari p su with an i as in ff re t rt i l COP lti g f om an i re se i GFR and FF Thus the p umed det rmi ant mean change i oncoti pr ur of th pillar and o pe itub l t titium

I d t vo d ia rp on should be mpha ed the the pe se com de en appl phy log l condi on l wh ch the p ox mal absorpt ve spac re f on sp l an ct nge GFR p unchanged or re duced ab orpt on at by h ra nal ubul an be man made ... should be equ lly we l aired at very low ma ked reduc on flow now he l moph on sequen re ve and by marked the reab ve at by he l op f Hen nd ro d l sequen

Al he sen ... dence th la pe change pe ub and rd on l p when they occu pe re as end fy rt in mode ... re imal epi hel um.

urrounding th p oximal convol tions in teady stat must be very small Evidence obtained in vitro as well a in vivo ugge t that the sensitivity of the proximal tubules to changes in oncotic pre sure f the rrounding medium i ather mode t El vati g th protein conce trati in th b th to twic normal in reased net w te and odium flux out of solated mi rope fused segme ts f proximal convolutions by about one th rd in Imai and Kokko xperiments (1972) while G antham t l (1972) were unabl t detect any ffect on absorption rate by hyperoncoti b thing solutions In both studies marked depre sion in et fluid t an f re lted when normal rum in th bath w replaced by completely coll id f ee ultrafiltrat A low se itivity to in reas in hydrostatic pres ur w also noted An nhibitory ffect on net t ansf by colloid free peritubular medi may not even be due to the reduction in COP B rate t al (1973) reported that replacement f b th serum by ultrafiltrat of homologous serum prepared with membrane xcluding sol te with molecular weights gre ter than 14 000 in contrast to ultr filtrates prepared with memb anes excluding solute with molecular weights gre t than 50 000 aused no dep ssion of fluid absorption Th uthors con luded that factors the than th oncoti pre sure were re ponsibl f observed changes in net fl d tran port Disregarding the mut ally conflicting re ults obtained with the split-oil drop method several mo recent results obt ined in vivo by peritubular microperfusion with lloid free pe fus t have fail d t demon tr t any ignificant ffect on proximal absol te r te of reabsorption (g Cong Bartoli and Earl y 1973; Bol gr ve and Sch ier 1975; Steven 1974b) In other tudi any onsistent correl ti n between the at of proximal reabsorption and the oncotic pre sure and/ the filtration f acti n ould t be detected (g Arri uri ta-Muchnik t al 1969; Ley ac Frederiksen and Skinner 1975)) and Blantz and Tucke (1975) were unabl t find any r-relation b tween the net i terstitial pre ure ($_i$ P_i) r th pas i dri i g f ce acros the proximal tubular epithelium and measured ab l te ates of proximal reabs rption in the teady tate of hydrope i nd f llowing volume xpansion with plasma line Finally f chang in the oncoti pr sure f the peritubul an ironment had any ignificant influence on net ab rption t th rat of reab rption hould be progre ively depre sed du i g p oximal tub l occl ion f llowing int rruption of the blood upply bec se f dilution by th reabsorbat Th evidence f cons an t f reab rption during the tubular occlusion an the quan t ve ag eeme t between reabsorption

rates measured und r f ee-flow conditions and tho calculated f on the OT provide strong argument gain t the physical factor hypothesi

Curr tly onsiderabl int st has been take in th po ibl achi vement of filtr tion quilibrium mainly because it i beli ved appar tly n ce ary to xplain th obs rv tion of pa ll l cha ge in en l blood flow and GFR without chang in the filtration fr ction nd th r by without change in ff ent rt i l r oncoti pr ure Within the framework f th concept in which the peritubula vironment ontrols the n t i osmoti fluid transport acros the proximal tub l by hydrost tic nd oncotic forc (Dean Robertson and Br nner 1973) ingl nephron glomerular filtr tion rat (SNGFR) will ary i proportion to change in plasma flow r t when filt ation p ur equilibrium occur (Dean Robertson nd B enne 1972) In ccordance with mi opunct r data (Daugh rty t l 1972) proximal tubula abs rptive rat hould remai un ffect d by pl ma flow-dependent v iation in SNGFR following g volume xpan ion with plasma which would l ave the ff t and ffer t oncoti p ure un changed Ap t f om th question ised above (p 15) concerni g the v lidity of the occur ce f filt tion equilib ium p u equilib ium appa ntly i n t obt ined gene lly Thu Blant Rect and Seldin (1974) found filtr tion quilib ium i on gr up f Munich Wista at but t i anothe r neithe Allison Wil on and Gottsch lk (1974) nor Kâll Fog t l (1975) found evidence f filtr tion equilib ium i thei Wi t o Sp gue Dawley at pectively; and filt tion equilib ium did t occu in the dog i ve tig t d by Knox (1975)

However in iew of the hyd odynamic i tanc f the entire neph on th ide that SNGFR an be di ctly plasma flow-d pe dent i equ lly logi lly inc ns tent th id th t p mary cha ge in SNGFR an ff t he post glomerul onco p and the by the proximal abso pt on Evo f f i tion equilib ium may be ached bef th ff t d f the glome l c pill und nd y nc se pl flow which move th po t f p eq l b um t d e ff t rt i l wo ld l ause an th ne f l t tion p s h h g pressure f llowed by ar immedi t and pa l nc se the p nal ub l p ur we ha e h d g f t f l ill not be cha ged p a i h SNGFR na s Til i y me lar t d p

the filtration pres ure can be dir ctly plasma flow-dependent The ame criticism lso pplie to the various othe models of glomerular ultr filtr tion dynamics recently publi hed (g St ve and Strõbæk 1974; Hus Marsh and Kalaba 1975)) though recogni ing the b illiancy of the quantit tive formul tions nd their value in det rmi ing filtr tion coeffi ients rte iolar re istance and the influences of th various parameters on the filtr tion pres ure these model re f littl value in predict ing which factors actu lly are th main ltimat det rminants of the SNGFR in th intact nephron in vivo (r ven in vitro) The model only desc ibe the vents and r conditions f presumed constancy of the proximal int tubul pre sure thus di regard ing th flow resi tance f the various tubul r segme ts The i k of mi l ding th reade by omitting consid tions of det r-minant sequence of vent would be l s if uthors of such model were more tringent in the use of the term SNGFR (o GFR) Rathe they should use the term glomerul filtr tion pre sure unl stimat have bee mad on th accompanying chang occu i g in th tubular pressure permitting valid quantitative val tion of th actual changes in th ff ctive filtr tion p ure and thei time course

Th xperimental finding by Daugharty et l (1972) th t SNGFR did in fact increase in proportion to pl sma flow following pl sma infusion without change in ithe ff rent oncoti pre sure or proximal absolut reabsorption te may be xpl ined by th conditions of their mi ropun ture experiment A lik ly rti fact i th t fl id f determination of SNGFR was llected in such fashion that the proximal luminal pre ure was maintained the ame unde the hydropeni control condition and f llowing volume expansion A more correct xpe iment would h ve been i th control period t collect th fl id unde ontrolled luminal pre su e equal to the free flow pr s re; then to infus the plasma and me re the rise in proximal lumin l p ure re lti g from the volume xpansion and fi lly t re-coll ct fluid t this ew intr tubular pr re

The eff t of na the i on the functional tate of the kidney Even though se ious h lleng h ve f iled t invalid t the OT-method so f it remained fact that othe i vestigators (Horst t al 1966; Lowit Stumpe and Ochwadt 1969) were unabl to reproduce the inhibit ry ff t of angiote in on p mal tubular function i vivo ob rved iginally i OT-experiments Al that

Wahl and associat s (1967) when using the OT-method found th occlusion time (and transit time) in ariant over la ge range of v i tions in GFR i contrast to the re lts obtained by Leys ac The finding by Wahl t l wer xpl ined by their me ured luminal radius quared which incr sed in direct p oportion to GFR Thu the rate of proximal r abs rption as lcul ted f om the change in radiu and the OT increased in pa ll l with GFR in accordance with the Copenhagen sult ; only in Copenhagen the OT (and TT) aried whil the radi wa constant ove the ame range of inulin clea ance The ult obtained in both laborat ries a internally consi tent. Thus the diff r nce in results wo p o- bably due to diff rence in the function l tat f the animal ther than to ro of me ur ments emphasized by Bojesen and Ley ac (1969) A differ nce in the choice of anae thetic wa uspected as a ause of the discrepancy because most inve tigator including those in Thur nd Gchw dt group used and till use the thiobarbit t Inacti whil Ley ac had l y used an oxybarbitu te odium amobarbital (Amytal); imila ly Ar iruri ta-Muchnik t l in Gottschalk labo t ry (1969) used an oxybarbit t sodium pe tobarbit l and found o ign f ignifi ant hange in proximal luminal adius ove pon taneous or induced v i tion i GFR Renal function in two large group f rats nae thetized ith I ac i and Amyt l pectively we the for ompa ed unde therwise ide tical conditions The lts disclosed th t th ame f ct on l patt rn as that de cribed by Wahl t l co ld be p oduced l by th Copenhagen g oup in at nae theti ed ith Inact nclud ng both the on tancy of the oc lus time Lime l 1972) and th adiu i tion (Kime t l 1973) P imal ab rption te ve ged 0 72 ml mi 1 p q f kidney weigh in he Amyt l ae theti d t and onl 0 46 ml m 1 g k 1 the Inacti g oup Al l t p oximal f ac onal ab pt dep sed i the I acti g p orp d abo 40 the Amyt l i The diff en q l gr f an th pr imal ab pt ve cul ting pla ma once t ti w l d t th plasma onc t l Ar ggested hat I act nh t s ns t e t ab p e capaci y i Ar T l t d by lts ob- t d ff fl id t nsport by b a Th ten en t l 19) fl id ab rption w f und mean con t t on

of both an esth ti s measur d in plasma of anas theti ed rats (about 5 10 ^{4} M) whe ea almost t times higher concent tions of Amytal we required for compa abl degree of inhibition At concentr tion of 5 x 10^{-4} M Amytal had n ffect on fluid ab sorption Furth rmore change in fluid absorption t by the gall bladde induced by Amytal were reversibl and accompanied by parall l change in oxyge onsumption In contrast I ctin induced depr s ion f n t fluid transf was i reve ibl and t accompanied by any hang in oxygen consumption A diff rent on lusion was ached by Dev t l (1973) in Munich who were abl to obtain dequ te anaesthesia with administr tion f omewhat smaller dose f Inacti (10 15 mg per 100 g of body weight (b w) than th t used by Elme t l (12 25 mg pe 100 g b w) Dev and his asso i t fo nd no diff ence i SNGFR in p oximal tubular reabsorp t on te between t anae th ti ed with Inactin and Amyt l; no could they d t ct any rrel tion betwee th proximal reab orptive r te and th dos of Inactin They concluded th t anaesth ti doses of Inactin did ot interf re with tubular function and that there wa n diff rence betwee Amytal and Inacti i this aspect How ve plasma barbitur t concentration we t measured; larger pplem tary d se f Amytal were give intravenously ther than intrape iton ally; and the umbe f animal tudied by De was small Hence the data are not directly comparable with Elme t l es lt Th problem was th fore re-i vestig ted by Ley sac nd hi group excluding fo th sak f comparison ll ts whi h ould t leep adequately with t tal dose of In ctin f t most 15 mg 100 g b w [1] (Ley sac Frede iksen and Skinne 1975) I ompl te greeme t with the d ta reported by Dev t al it w found that within thi narrow ange of Inactin dose (12 5 15 0 mg pe 100 g b w) p oximal fr cti nal reab rption was lightly but sign fic ntly high than previously ob rved Moreove ab ol t rat f pro imal absorption me ur d in Inactin anaesthati ed ts were withi th ang of alue obtai ed in th Amyt l se i) and the w det ctabl corr l t on betwee th te f ab sorption and the dos f Inactin admini tered Howeve ompari on of the d t obt ed in the Inacti anae theti ed animal with thos ob ed Amytal anaesthesi howed ignifi ant dif f renc I the a group th ang f pro imal reab rption t was na ll lies be g in th lowe half f the ange f values meas Amyt l na th ti ed t ; d more important a mode l ad give intreve ou ly (equal to 1% of b w) failed tc h p oximal reab rptive apacity i the Inacti g ou l volume xpansion with line

sed highly ignificant increase (on an ve age by 27%) in proximal tubular function (and GFR) i the group f animals ana thetized with Amytal Thus even low dose of Inactin appe to inhibit and limit the proximal reab rptive apecity such th t the esponse to physiological timulus is abolished o at lea t blunted

Anothe diff rence between Inactin and oxybarbitur t anae th ti ed rats wa reported by Steven (1974) in a micropunct re tudy Unde condition f odium pentobarbital ana the ia 3 mm Hg reduction in proximal intr tubular pre sure achi ved by ont lled suction aused ignificant increase in SNGFR where in I actin anae theti ed r ts imil r reduction in luminal pressure we unabl to ugment th SNGFR becaus the lume coll psed t l ast par-ti lly proximal t th collecting pipette Th observ tion in-dicates that the proximal tubul w ll w profoundly changed by Inacti being more ll psabl ompared to tubules unde con ditons of oxybarbitur t anaesthe i which would xplai why o-v i tion f the proximal lumin l radius with GFR has consi tently been found over the tire ang of filtration te i tudi on Inacti anaesthetized at Finally it has most recently bee hown th t I actin i ontrast t oxyb rbit t impairs the utoregul tion of RBF both in t and dog (Cong and Burk 1976) Thus the choice f the an theti I acti fo tudi on proximal tubul function and it regul tion would ppe to be mo t unfortun t in p ticular f t ting an i hibitory ff ct f angi tensi (Ro te t l 1966; Lowitz t l 1969) whi h ha been detectabl only t the highe t pon taneou tes of proximal absorption obt ined i Amytal nae the i

The es lt of those tudi whi h oem o solve long-lasti g controversy empha i that the ff ct f th anaesthetic its lf hould be tak i to on ide tion when val ting the lit t and that ve un cogni d ff ct of th anae thetics mu t be matt f continuing conc rn as mphasi ed lso by Las it (1975)

Effect f angi t n in on i amo ic tran pith lial transport There i increasi g idenc tha angi ensi II may influence t ansc ll l t anspo t f odium and w t i t ue pitheli pr umably by direct ff ct Da i Mundar d P r ons (1970) de on tr t d imul ti g ff ct f angi in II (10^{10}g ml^{1}) on int tia l fl id f y i verted a prepar tions in vi ro I t ipped loni muc t on t se t ed by pre ious neph

rectomy and adrenal tomy angioten i has dose-d pende t biph ic action on t fluid t ansf r; low conc tr tion (10^{11} 10^{12} g ml ^{1}) stimul ted whilst highe concentration (10^{9} 10^{8} g ml ^{1}) depre sed net fluid t an f r (D vies Munday and Parsons 1970) Th se l tter observations were confirmed by Hornych Meye and Millie (1973) who also howed that the biphasi respon to angiotensin was de pendent upon the previous nephrectomy-ad nalectomy Angiotensin used an increase in odium nd w t t fl x acros muco a from ascendi g olon of n rmal rat whereas it inhibit d net fluxe acros mucosa from d sc ndi g colon at ll dose level (10^{11} 10^{6} M) In l ter tudi by Bolton and h r associ te (Bolton t al 1975) a biph i ff ct of angi ten in on j junal net fl id absorption in vivo w demonstrated in normal rat ; ag in ubpresso dose (0 59 nano-g kg ^{1} min ^{1}) stimul ted whil high pre o doses (5 9 nano-g kg ^{1} min ^{1}) inhibited ab orption Evidence that th timulati g ff t of angiotensin on olonic t ansport apparently d pends on pr tein ynth i t the t an l tional t ge h been provided (Davi Munday and P rsons 1972) gg ti g that the mechanism of this effect diff p foundly from th rapid in hibit ry ff ct of gioten in on proximal tubular function (Leys ac 1964) An inhibitory effect of angioten in II on in itro fluid absorption by gall bladders from ormal abbits was reported by Ley ac and hi g oup (Leyssac et al 1974); th onset f thi ff ct was pid and the maximum ff ct was obt ined within 30 min t Any timul ting ff ct of angioten in could ot be de tected in thi prepar tion The ff ct of angiotensi on the g ll bladde wa due to inhibition of th active component of the trans f proce lea ing the pas ive rosal to-mucosal flu of odium and the pot ti l diff rence unaffected Ohmi re istance inc ed lightly (by 10%) I mi rope f ion t di on isolated segments f proximal tubules in vitro Burg and Orl ff (1968) were unabl to d tect y ignifi ant ffect of angiot nsi (2 10^{6} M) on net fl id tran f Howeve et fluid transf at were les in vitro tha in vivo d th dose applied was tremely larg oithe f which factors might h ve concealed pote tially phy io logi al ffect

More important in thi ont xt therefo e was micrope fusion tudy by St vo (1974b) i which the inhibitory ff ct of angio t in on proximal tubular reabso ption wa eproduced in vivo in pentobarbit l an theti d t Lat pro imal tubul fl id wa coll cted t f flow lumi l pre ure lt rn t ly from dif f rent tubul d g pe tubul apill ry mi roperf ion t

20 nano-l min $^{-1}$ with angiotensin cont i ing solution (20 nano-g ml $^{-1}$) or with saline Absolut fluid reabsorption rate w ignificantly d pre sed by 7 4 nano-l mi $^{-1}$ g k w $^{-1}$ and tubular fl id/pla ma in lin rati ignificantly decreased by 0 79 during angioten in perfusion Peritubul mi roperfusion with aline had n ffect on proximal r absorption rat as compared to value obt ined with out pe itubular perfusion in the ame kidney Th di ect ffect of angiot in on isosmotic fluid transport by va ious pith lia in itro and in vivo has been demonstr ted; and it inhibitory eff ct on p oximal tub l r reabsorptive pacity has been di ectly ve ifi d tilizing different methodology than that used f it fi t demonstration The invers relationship betwee log pl ma enin concentr tion and/ r activity and the p oximal ate of r ab orption f und in Amyt l anae th ti ed r ts over the tir ange f absorption rat (and GFR) i luding th range occur ri g ponta eously (Elmor t al 1972) well th t induced by mode te s line loading (Ley sec t l 1975) i con i tent ith the hypothe i that the endogenous cti ity of angiot in modulate proximal abs rption ate unde these conditions Moreove such an inverse rel t on hip as absent i rats anae thetized with Inacti which w shown t inhibit nd limit th p oximal abs rptive function

Th int enal v scul effect of endogenous ngioten in II

Within the f amework of th clas i al theory it might be con iv- abl that ndogeno gi t i could pl y ol qul tion f GFR and RBF by an tion p edominantly on th p -glomerul art i l ince accordi g to thi oncept be t of filt tion i almost xclu ively determined by the filt tion p re (ften f rred t glomerul dyna i Thus Thurau ad oc te the ew that the acti ity of th i angi ten sy t m i gul ted by feedback mecha i n i the macul de i uch w y th t l ated ly di tal odium on tion mor l k ly sodium abs rption at the it f ma l de timul t th i y ten (g Th 1972 Thur and Mason 1973) Thus g p mary dec se i tubul functi (nece ily t it p ox mal t the mac l de) by incr as g th di t l NaCl-concent tion nd/ fl id flow t would t sul t the juxtaglomerul rp t i it f loc l ang tens f rm t on th ff ent t i l ould be onst i ed b by dec g GFR nd tab- l hi g normal fl id delivery t th d t nephron gments h d ct id n f om r ope f on tadi th t the t f ab rpt on by l d l mb f Henl and conceivably

lso by the early di tal tubul i directly load-dependent probably by gradient limit tion (g Anagnostopoulos Kinn y and Windhager 1971; Kunau Webb and Borman 1974) Thu any acut in re in the delivery f fluid to the loop and th macula den should timul te th renin system Contrary to this view i th well known f ct that such an increase in loop and di tal tubul fluid flow used e g by an acute intrave ous aline load de pre se renin rel within few min te (f g Leyss e t l 1975) whil udd n reduction i filt tion pres ur and th eby in tubular pressure and flow te timul tes renin rel as immedi t ly and contin ou ly even in th isol ted perfused k dney (g Hofbauer t al 1974) Aft period of reduced filtr tion pres ure and ischemia ly distal sodium concentra tion i in eased indic ting impai d function of th tubular epithelium as shown by Thur u t l (1967) in particular that of th diluting gment i e the ascending limb f Henl ; most lik ly th refore the reab rptive capacity of the macul densa lls hould l o be impai d unde thi conditio which should depres nin rel se according t Thu an concept; but it seems to timul te the rel ase according to measurement

Apart from thes pparent difficulti fo accepti g Th re view Gros and hi associate have obtained indirect vidence of an ctio of dogenous as well f xogenous angioten in predominantly on the post glomerular art iol U ing an i lated pe fused kidney-p eparation these inve tig tor found marked increas in GFR and th filtr tion fraction f llowi g ithe addi tion f enin ub trate to ub trate-free perfusion medium (Krah H fbaue and Gros 1970) or addition of angioten i II to th medium (Krah H fbaue and Gros 1971) Similar incre se te of GFR and FF were port d by Hulme and P in (1975) fter infu on f ithe enin angiotensin i to the sy temi ircul tion of at wh reas equipressor doses of no adrenaline o adrenaline had b t little ff ct on the FF and GFR More direct e idenc of a post glomerular vascular action f dogenou angioten in has ecently bee obtained by St ven Pe itubul mi rope fus on of ngioten in II cont ining perf sat (20 nano-l min [1]) used dose-depe dent increas t in th luminal hydrost ti pre ur f adjacent proximal convol tions angi g from 1 to 6 cm H_2O i pe tobarbital anae thetized r t (St ven 1974b) Since the intratubul pr sure closely refl cts acut change in the fil tion p sure th me sured pre ure incr as could be due ithe t pre-glomerular v sodil t tion or t post-glomerular

v soconstriction Primary arteri lar vasodil t tion caused by angio-tensin has never been obs rved The r corded pr sur incr ase must th ref e have been due p edominantly to fferent vasoconstriction It should be mentioned that the magnitude of uch r sponse to ngiot nsin will depend on the p -existing ff ct on the ve sel by endog nou vasoconstrict r sub tanc ; hence it should be mor marked under conditions f high function l activity a may be obt ined i he lthy rats anae thetised with mi imum do of oxy-barbitu te

I mo r cent tudies it was shown th t pe itubula microperf ion with th competitive angioten in an log 1 r-8 al angi tensin (8 r la in) c used the oppo it r ponse i a ignifi ant intra-tubula pr e d op of 4-6 cm H_2O i dicating po t-glomerula va odilat tion (Steven 1976) Thi latte es lt is crucial howing firstly th t the vascular re i tance *in ivo* is ontr lled by ndoge ou giot in; secondly th t it i control p f n ti lly on the ff ent art 1 In con lusion the observ tions indicate th t the p imary v scul action f endogenous ngioten in i p f renti lly po t-glomerul onst i ti which will tend to le te the glomerul r capill ry hyd i pre sur (i the filtration p) moreove the post glomerular va cula e-i tance *in vivo* i ontroll d rmally by th i t ar al activity f the reni angi ten i ys em i g eement with the p ent working hypoth i

Tubulo-glomerul feedback

Conside abl ffort h ve bee tak t t t p iou ugge tion f the ope tion t the gl neph on i el f an i t en l f edback co t ol f GFR and fl id delivery t th di t l t bul modi t d by th juxt glome l ppa t (JGA) Mo t con inci g vidence pport g thi hypothe f t ubt l ed by Schn mann nd h oc Sch rman t 1 19) legant techniq f lly k g ll po bl p w l n d cogn) tif demon d w l 1 ll ry p do sed d g mi ope f on h i H i i bove 15 and 1 1 T i ithe p glomerul on i dil m h be pe edly d i tud by Schn mann d t l l 4 Müll uu 1 l Ab ed lun d by ope mann t l olium 115

Schn rmann P rs on and Ågerup 1973) or with diuretics known to interf re with loop and di tal tubula lectrolyte reabsorption (Wright and Schn rmann 1974) suggesting th t the feedback re ponse depends upon th transepith lial transf r of lectrolyte rom th macula dens Al o it ha been hown that alcium i nece sary component of the perfu ion fluid for the feedback re ponse to occur (Burk et l 1974) The response appear d to be asymmetri sinc chang in loop pe fusion rat below the predet - mined normal l vel had no detectable influence on the filtr tion pre ure as estimated ithe from measurements of SNGFR llected t experimentally fixed intr tub lar pre ure (Schnermann t al 1970; 1973); from measurements f proximal int tubular pre sure change (Bierhol t al 1974); or from direct measurements of glomerular capillary hydrostatic pres ure (Blant t l 1972) Further indirect evidence that th feedback response to in reased fluid flow i to the di tal tubul involve the renin-sy tem has been obtained Dietary alt intak modified the sensitivity of the feedback mechanism; salt loading which educes the re al renin content and release dimini hed the response (Dev Drescher and Schnermann 1974); blockers of the renin angiotensin y tem (sar lasin and converting nzyme inhibit) also blunted th response reported by Schnermann (1975) Direct vide ce indicating that the renin system is involved in the feedb ck mechanism has not been *obtained*

The operati of uch feedback sy tem unde n rmal hydropeni conditions predicts that oil blockade of l te proximal convolution and thereby f fluid flow int the arly di tal tubule should l v t the filtration pre sure Proximal llect ons f fluid t free flow pressure for measurement f SNGFR hould therefore give value f SNGFR higher than those obtained in th ame kidney by llections of fl id from di tal tubule Thi prediction has been confirmed in several tudies (g Schnermann t l 1971; Davi Schn rmann and H rste 1972; N var t al 1974) whil othe inve tig tors h ve been unabl to det ct ch diff rence in SNGFR depende t on th it f fluid coll tion (g Morgan 1971; Bartoli and Earley 1973; Knox et l 1974) Taking int consideration the tech ical difficultie nd i k f rtifacts inherent in micropunct t chnique th i fluence on the f ed back ystem f previous lt intak and of the phy iologi al t t of th animal during the xpe iment including lso th ch ice and dos of anas theti it i h dly urp i ing the conflicting result have been reported llect vely these studi do provide trong

and di t vidence indi ating the xi tence of a feedback control mechanism whi h senses changes in a flow dependent ignal t the level of th ea ly di tal tub l and which in respons adju ts the glomerul c pillary hydrostatic pr s re

The que tion of how change in delivery of tubul r fl id into the arly di t l tubul i sensed and conve ted to an ffector r pons remain a matter of pur pecul tion

Evidence has suggested firstly that transepithelial transf of lectr lytes acros the macula densa may be goal f ignifi ance; secondly th the reabsorptive t f this tubular segment is directly flow-dependent Recently studie on eni release in vitro (Frederik en Leyssac and Skinne 1978; Baumbach Leyssac and Skinne 1976) have indic ted that the renin containing juxtaglomerular cells may respond t small osmoti challenge with pas ive change in l rele se function of ll volume unde thei in tro conditions Based on the hypothetical as umptions firs ly that the tubulo-glomerula feedback i medi ted by the enin-angiotensin system; secondly tha the passive cell volume-dependen renin leas observed i vitro may be f phy- logi l importance in regul ion f ba al eni rele se in vivo these f nding sugge ted to the uthor unifying hypothesis accord ng this hypothesi — one among seve l po ibl the juxtaglomerular cell (JG-cell) could func on systemi parameters whil the same time cell volume-sec ion coupling could unde lie both the baroreceptor nd chemoreceptor concepts of enin rel ase (cf review by Davi and reeman 1976) and accoun f feedback re ponse Thus th hypothesi predicts that an incre sed te f sod um and chlo de reabsorption by he macul densa f llowi g inc ased del very f fl id to th tubula segme would inc ease the loc l te titi l fl d osmolali y Th hyperton y in urn would cause cell shrinkage and he eby dep rea l se

Summary d con luding r m rks f figs 1 3 and 3)

1) It h bee abl h d h th p oximal ab rpt ve pacity i p ima y abl depe d f FR (th filt ed load) f i t lum l p d l lum d anet I h le d prov d h he al p k) thi neph n dmo t onst al l i le

2 l dens t the p ox l ub) r m p ima ily i l i se i P_T) Aft lu. l p pul t d t wt h ma t

tates It has been di proved that th proximal lumin l pre ure is onstant parameter

3) I h bee shown indirectly well as directly that the pres sur drop across th glomerular membran - th t ultr filtrati n driving f rce ffective filtration pressure - is low in the order f 10 15 mm Hg or l Hence small changes in proximal intra-tubular pre ur all th fact rs being equal will ca se large hang in GFR.

4) A tubulo-glomerular feedback mechanism which responds to change in di tal fluid flow d l very with hange in th filtration pre ure h been demonstr t d directly

5) It has bee hown and l tar ve ified with diff rent direct techn que th t angi tensin II has presumably direct tubular action whi h inhib ts th proximal reabsorpt ve c pacity of isos moti flu d

6) D rect evidenc h been provided indi ating that the vascular ffect of endogenou angi tensin i p edominantly an ffect on the eff rent rteri l ; and th t the esistance of thi arteriol i contr lled i vivo by th local tivity of angi ten in II Thu the tw intrare al ff ct of angiotensin act in the ame dir ction on the proximal intratub lar pres ur

It would seem, th ref re that th fundamental as umption f the pre ent working hypothesi as well its impli tions h ve bee verif d and tablished Furthermore ach link in the chain of ve ts in the hypothesi has been confirmed by results obtained in several laboratori and by utiliz tion of var ous technique On thi background th term worki g hypothe i may now be di mi sed in my opinion; rathe t should be reg ded as new theory replacing the disproved and more row las ical theory

Th new theory i i complet harmony both with the an tomi l tructure of the nephron and with the speci l local tion of the re in ystem I addition it ff rs impl explanation f th larg development f th proximal tubular segment i mammal

Homoithermi as mammal are with high blood pressure and ascular system with art iol which react to many timuli un rel ted to the salt and wat balance thei filt tion pre ur will be rel tively un tabl arying ove ange f seve al mm Hg Accordi g to the theory th proximal tubular segme t of the nephron and th re i syst m functions i part t l ast by means of feedback mechanism b ff sy tem which an apidly tabilize

th intr tubular pre ur in pite of va iable propulsive pr

In thi fashion a table delivery of tubola fluid both to the loop of Henle and to mo distal tubular segments i n red; and it n be asily understood th t large pre ur change can be compens ted f with l rger proximal absorptive pacity and variability of the ff rent t riol r re i tance

A tabilized fluid flow to the segment di tal to the proximal tubul i da i ive nece ity if lt and w t r b lanc is to be maintained partly bec us the optimal function of th conce tr ting mech nism i the loop f Henle d pends upon th maintenance f tubul flow tes within l tively narrow ang ; and partly beca se th absorptive c paciti of di tal tubul and coll ting duct by which the xcretion of lt i finally r g lated i small nd lowly adjustabl (g by miner loco ticoids)

Thus withi the f amework f thi theory th renin sy tem and th p oximal tubol segme t play a central ôl f r the comp hension of th mai tenance f body salt and wate balanc and hence of rmal blood p sure B t it should be t ngly emphasized that quit identiy we do not k ow but a few of the p obably many fact including i t a en l facto whi h may po ibly regul t lt xc etion; and th ignificance f othe fact rs in part unk own perh ps which may influence and limit the proximal re- ab rptive pacity unde arious the ondition also mains undefined But the ba is for f th r p ogr in thi fi ld may be imp oved I hope Ob iously the new theory has a bea ing l o upon pathophy i logi l tat Hopef lly it may influe ce futur re rch on the pathogene i of hypert ion and dise se uch as t renal f il i positive di ction

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Muscle Strength, Fibre Types and Enzyme Activities in Man

BY
ALF THORSTENSSON

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Muscle Strength, Fibre Types and Enzyme Activities in Man

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INTRODUCTION

The present study is an attempt to relate the histochemical profiles and biochemical characteristics of human skeletal muscle to its mechanical properties

The speed of muscle shortening is known to be inversely re lated to the load against which it shortens This basic finding in muscle mechanics was first made by Fenn and Marsh (1935) and formulated in mathematical terms by Hill (1938) This force velocity relationship applies to both smooth and striated muscle (for refs see Sonnenblick 1962) even human elbow flexors during voluntary co tractions (Hill 1940 Wilkie 1950 Asmussen et al 1965 Komi 1973) In these studies either resistance or velocity was kept constant A new method has been de igned based on Perrine s work (Perri e 1968) i which different constant velocity levels are easily achieved The force output of different muscle groups during ma imal contractions can then be conveniently studied at different muscle shortening velocities

More than a ce tury ago Ranvier (1873) observed differences in the force velocity properties of rabbit skeletal muscles of differing gross morphology Li ks between the co tractile properties of a muscle a d its metabolic and morphological characteristics i terms of the distribution of distinct muscle fibre type were firmly established s bseq e tly (see Close 1972 and Burke et al 1973) E periments initially involving histochemical analyses of whole muscles with fast or slo contraction times and later direct t dies of si gle motor units led to the conclusion that motor units ca be cla sified into two main types fast twitch (FT) a d slow twitch (ST) (other omencl tures are also used; see Close 1972) The muscle fibres belo gi g to each type of motor unit have bee sh w to be homoge o s (Edström a d Kugelberg 1968 B rke et al 1973) a d can be co ve iently ide tified by histochemical means (s M th dological Aspects) Ma imal tetanic te sion appears to be higher f r FT motor nits than for ST nits eve when the t nsio is l t d to it cro s sectio al area (see Burke a d Tsairis 1973 Bar y a d Close 1971) D e to methodological difficultie this l tter differe ce is l ss well est blished (cf Close 1972) The F mot its display a high r s ceptibility to fatigu upon

repeated electrical stimulation than ST units (Edström and Kugelberg 1968 Burke et al 1973) However the fatiguability within the FT motor unit pool varies and appears to be inversely related to the oxidative capacity of the fibres These differences led to a subdivision of FT fibres into two types one which is comparably resistant to fatigue and another which fatigues rapidly (Barnard et al 1971 Burke et al 1973) Both types possess a high glycolytic potential in contrast to ST fibres which have a low glycolytic and high oxidative capacity

For obvious reasons much less detailed information is available about motor units in human muscle than in animals However autopsy (e g Johnson et al 1973 Edgerton et al 1975) and biopsy (e g Engel 1962 Gollnick et al 1972) studies have shown that most human skeletal muscle consists of a mixture of muscle fibre types histochemically classifiable as ST (type I) or FT (type II) fibres Metabolic studies of human skeletal muscles using both mixed muscle homogenates and single muscle fibres have essentially confirmed the findings from animal muscles (e g Schmalbruch and Kamienieck 1974 Essén et al 1975) One striking feature of human skeletal muscle is the inter individual variation in the proportio s of FT and ST fibres within any given muscle Data on the contractile properties of human skeletal muscle are limited Two types of motor units were postulated on the basis of the twitch contraction times recorded for single motor units (Sica and Mc Comas 1971); a slower contraction velocity has been reported for fibre bundles containing a high percentage muscle fibres rich in mitochondria (presumably ST) (Buchthal and Schmalbruch 1970)

Co tractile activity in muscle is controlled by regulation of the Mg^{2} stimulated ATPa e (adenosine triphosphatase) of the actomyosin of the myofibrils (Perry 1974) The validity of Mg^{2} activated actomyosi ATPase in this respect is supported by most in vitro studies (see Barany 1967) A high correlation exists between the speed of contraction and activity of Mg^{2} activated ATPase in both myosi and actomyosin (Barany 1967 Barany and Close 1971) The activity of this enzyme may therefore be one of the controlling factors in ontraction velocity At present it is not known whether or not this enzyme is related also to muscle tension e g by controlling the number of activated cross bridges

The fast ATP hydrolysis during vigorous muscle contractio s calls for rapid resynthesis of ATP This can be accomplished from local stores of creatine phosphate via the reaction catalyzed by creatine phosphokinase (CPK) (for refs see Karlsson 1971) A higher level of activity of this enzyme in FT muscle fibres than in ST fibres has been reported for rabbit and human skeletal muscle (see Patte and Bücher 1963 Sherwin et al 1969) The myokinase (MK) reaction transforming 2 ADP to ATP and AMP can also provide this rapid ATP reple ishment (Cain and Davies 1962) However the link between the activity of this enzyme and muscle contraction has been less firmly established (Noda 1973) The characteristics of the MK reaction are likely to be of relevance in the regulation of the rate of glycolysis since the ATP:ADP:AMP ratio is known to regulate the activity of the key enzymes glycogen phosphorylase and phosphofruktokinase (see Atkinson 1968)

More detailed information about the action of an enzyme can be obtained if a analysis of isozyme distribution is combined with determinations of total enzyme activity Isozymes of several enzymes including CPK and MK ha e been demonstrated in human muscle tissue (for refs see Watts 1973 Noda 1973 and Markert 1975) These multiple ariaties of an enzyme cat lyzing the same reaction are h iou ly eeded to enable the cell to cope with arious metabolic conditions Thes conditions may occur at different sites within the same cell or in different cells e g FT and ST muscle fibres A good e ample of this has rece tly been demonstrated for lactate d ydroge se in human skeletal muscle (Sjödin 1976) Muscle fibre spe ifi ity CPK isozyme distrib tio has been indicated for i s el tal m scle (h rwi et al 1967) MK has not been m d i thi respect

p ssibility of i terco ersion of FT a d T muscle fibres by p ysi gi al me s s ch as traini g is c rrently the subject f s i t p im tal m s ip latio s su h as cross in rvation i im latio a d te ot my ca l ed to changes in co r p rt s of f t a d slow muscles and co comitent altera t s i i (ee Close 197 Lowl et al 1974) However p s al i g s ot yet bee sho n to affect the isozyme p r e my fitrillar ATP se forming the basis for the classi

fication of FT and ST muscle fibres (for refs see Burke and Edgerton 1975) There is much evidence in support of the view that the distribution of the two main fibre types is largely governed by genetic factors (cf Komi et al 1978) The predominance of one fibre type in a given category of athletes e g ST fibres in endurance athletes (Gollnick et al 1972) would then be the consequence of selection However long term effects for systematic training can not be ruled out completely at this stage

The metabolic properties of a muscle can be altered by physical training Endurance training has been shown to cause substantial increases in the oxidative potential of both animal and human skeletal muscle (for refs see Holloszy 1973 Saltin 1978) These oxidative changes appear to be confined mostly to the ST fibre type and a selective hypertrophy of this fibre type has been observed with endurance training (Gollnick et al 1973) Evidence for an increase in the oxidative capacity of FT fibres has also been noted (e g Barnard 1970) Changes in the isozyme patt rn of lactate dehydrogenase after endurance training have been found for both fibre types (Sjödin et al 1976) No information is available on corresponding muscular adaptations in response to strength training

The main object of the present series of exp riments was to investigate the relationship in human skeletal muscle between con tile properties such as force velocity and fatiguability on the one hand and the quality of the muscle in terms of its distribution of FT and ST muscle fibres and the related activities and isozyme patterns of Mg^2 stimulated ATPase CPK and MK on the other hand plus the adaptation of these muscle properties to strength training

METHODOLOGICAL ASPECTS

Subjects

Subjects in studies I and III VI were recruited among healthy habitually active men mostly students of physical education In one study (II) Swedish elite athletes in various sport events participated along with a group of sedentary subjects with no experience of systematic physical training In all cases the subjects agreed to participate after being informed about all aspects of the experiments Most of the subjects were between 20 and 30 years of age (total range 17 39 Table I) Although muscular strength is known to vary with age the age variation in the present material was so small that it was considered to be of no significance to the results Some anthropometrical data for the groups of subjects e amined are presented in Table I Muscle strength was mainly measured as torque (see below) which is theoretically proportional to L^3 (L linear dimension) To circumvent the influence of body dimensions on the strength measurements the muscle strength w s expressed per kg of body weight (also proportio al to L^3) or in relative terms e g as a perce t of ma imal isometric strength (for refs on age and dimensio s see Astrand and Rodahl 1970)

Statistics

Co ve ti al statistical methods were employed to calculate standard deviatio s (D) standard rror of the mea s (SE) and linear corre latic coefficie ts (r) Determi ation of linear regression was performed i g th method of least squar s Intrai dividual differ a d differe es betwee m an values w re tested for sig ifica e i g depe d t a d i d pe dent Student s t tests r pe ive y The le el of sig ifica c i d noted with p v lues T meth i r or a defi ed as t e D f r a si gl e periment cal ul ed rom d uble det rmi atio s cc rding to the following q ti

$$\sqrt{(d\ d)\ /\ (\ \ 1)}$$

i hi d t e diff re c tot n d l al os d the me n ui f r d mb r of d termi ti s

In the text the method error will be expressed as coefficient of variance (C V) i e SD/x x 100 where x the mean

Table I: Characteristics of subject groups in studies I III and VI Mean values and ranges are given

Study	n	Age yrs	Height cm	Weight kg	Category
I	25	24	178	70 9	habitually active men
		17 37	165 191	51 7 95 0	
II	9	24	187	77 8	jumpers and sprinters
		19 34	181 194	73 0 86 0	
	8	21	178	69 4	downhill skiers
		19 25	174 181	67 0 75 0	
	7	27	178	64 5	competition walkers
		17 36	175 187	61 0 67 0	
	7	25	181	68 5	orienteers (cross cou try runners)
		22 29	174 185	63 5 74 5	
	10	26	184	75 1	sedentary men
		22 29	170 190	8 1 95 0	
III	10	30	180	69 5	habitually active men
		25 39	165 185	62 5 77 0	
VI	14	24	179	74 2	habitually active men
		19 31	169 186	59 6 89 3	

Strength measurements (I III)

Maximal muscle strength in the left knee extensor muscles was recorded as torque output during maximal voluntary knee extensions with constant angular velocity The subject was sitting in an experimental chair and the lower leg moved the lever arm of the isokinetic dynamometer (Cybex II Lumex Inc New York) The pivot point of the lever arm was aligned with the knee joint The angular velocity was controlled at the pre set level by an internal resistance which accommodates to the muscular force applied (see Perrine 1968 Hislop and Perrine 1967 Thistle et al 1967) The experimental setup is illustrated in Fig 1 and the different components are described The range of motion encompass d knee angles from 90 to 0 degrees[1] Isometric strength was measured at selected knee angles whereas concentric strength was recorded o er the whole range of motion Angular velocities could be selected between 0 and 180 degrees/s[2] Two attempts were allowed at each knee angle and velocity and the highest alues were noted

A r presentati e recordi g of torque output[3] during a maximal isoki tic o tra tion is shown i Fig 2 Peak torque is identified a d will be th pri ciple measureme t used for comparative analyses of mu le stre gth One advantage of assessi g muscle strength by mo s of torq e o tp t i additi n to its fu ctional connectio is the i rele a of the lo atio on the b dy segme t at which mo r mo ts re take The length of the le er arm was therefore kept c sta t i the prese t serie of experime ts However the l o gr s f the k e joi t could ot be ccounted for Nor were i d id l diff r i this or other a tomical parameters e g l gt f t e p t llar t do id red

r l a ili y d v lidity f the is kinetic dy amometer has c rted b high (Moffr id et al 1963) Torque alues dis

---

1) s g r press d i d gr s (90 d grees 0 5 π radians) i g f lly e de k e joi t

A r r l lt, i ref rred to l degr s/ (180 degrees/s π i /

3) c ig th it f i n 9 81 kpm



Fig re 1 Th xp rime tal tup f tre gth meas reme t
A adjust bl h i ith uppo t f houlders hip nd l gs
B i ki ti dy amomet (Cyb II) with ve t ma ke d
leve rs C p d l t D k f mplifi rs E UV-
record r (H eywell 201)

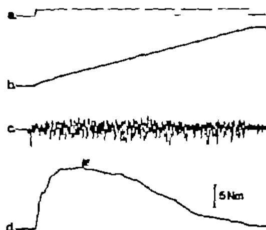


Fig re 2 Repres t t ve re rdi g of ma imal i i ti on
tr tion t t ng l vel ty f 60 degre /
eve t g t very 15th d gre f mot b pot t met
re rd g f ng l l ity EMG ignal re rd from th
cont t g astus l t li d t rq re rdi g d tes p k
t rq

played a lin ar increase in response to i creasing weights attached to the lever arm for calibration The test retest reliability co efficient for rep ated pplic tions of various loads was r 0 995 (n 70) M ffroid et al al o reported high values for the con st ncy of velocity (r 0 985 n 34) which was confirmed in the prese t study for the greater part of the rc of motion At onset of motion there i an unavoid ble time lag before the pre set constant velocity is reached This time lag (0 05 s) was large t at th high t velocity (180 degr es/s)

The reliability of subject ability to produce maximal torque at each velocity and kn ngle was investigated in 3 diff rent situations a) in consecutive attempts b) in randomiz d ff rt interrupt d by contr cti n at oth r velocities c) as in] but on two separate d ys These investigations produced the following over-all coefficients of varianc (C V) for the whol torque curve i e from 7 knee angles in th arc of motion and at 6 different velocities 6 4% (n 6) 6 5% (b n 9) d 13 7% (c n 6) resp ctively The C V for peak torqu prod ced a rding to a) ranged from 3 2 4% in a total f 38 ubje ts Th r lts were s pplemented by st dy f 25 h bitually active healthy me (g d 17 27 yrs) who completed the t t programme according to c) The C V for p ak torq e (the highest val es re ord d on two s p rate d ys) in thi group ra ged from 4 1 6 5% d f r subseq ent pe k l es the same ion 2 1 4 7% No sy tematic diff rences betw th two testing s s ion i irrespectiv of the programm (b r c) were fo d

Th i flue ce of fatig aused by the testi g pr ced re was h ked by repeating th i itial t t p ed ft r the completion of th entire programme 10 s bje t No sy tematic differe ce was f d b twe thes d t rminati n Th th eff ct of fatigue i nsid red egligibl with th present test battery in l di g up t 22 ma imal co tr ctio s t vario s peeds i terrupt d by rest interv l of 30 45 s

Mu lar fatig e w a sessed i perime t with repeated ma im l contr ctions performed n the i okinetic dy amomet r Angular v l ity was s t at 180 degre s/s Th subj cts were instructed to perform repe t d e t sions with maximal effort and to resume the tarting positio pas ively b twee ach contraction Every con tr ction lasted 0 5 s nd the passive ph s approximately 0 7 s i e an average of abo t 50 c tractions was performed per min

All subjects were tested on two separate days one day performing 50 and the other 100 knee extensions The averaged peak torque for the 48 50th contraction in percent of the corresponding value for the initial 3 contractions was taken as the fatigue index The C V for this measure was calculated at 3 2% (n 10)

The maximal knee extension velocity was estimated from the time interval between microswitches at 7^c and 15 degrees knee angle during knee extension against no external load The best of 5 attempts was used Day to day variation for this measurement amounted to 5 2% (C V)

Strength training (VI)

The training programme consisted of exercises used by athletics to achieve optimal improvements in maximal strength The programme included mainly ma imal or close to maximal exercises (3 sets of 6 repetitions) for the leg extensor muscles carried out in the form of squats i e knee bends and extensions with w ights on the shoulders The load was progressively increased over a training period lasting 8 weeks (3 sessions of abo t 1 h per we k) The programme also included various jumping regimens e g vertical jumps The effects of this programme was tested by a v riety of ans P rformance tests comprised one repetition ma imum in squats ve-tical jump standi g broad jump and determination of ma imal isometric force during two leg extension (Karlsso and Ollander 1972) The isoki etic devi e was not used in this study

Muscle sampli g (I VI)

Skeletal muscle samples were obtained from resting vastus lateralis muscle using the eedle biopsy technique a described by Bergström (1962) The vastus lateralis was chose because it a a major con ributor to the force ge erated duri g knee e te sio (cf Fig 2) and is also located at a site co venie t for muscle biopsy sampli g The other muscles e gaged in the ge eration of measured total kne extensor strength were ot biopsied In one of the studies (IV) surgical biopsies were obtained from hu-a papillary and soleus muscle for compariso of isozyme patterns (see below)

Histochemical methods (I VI VI)

Muscle sp cimens for muscle fibre typing and area determination were trimmed of fat and connective tissue mounted in histoclad and frozen in isopentane cooled with liquid nitrogen When not analyz d immediately samples were stored in a freezer t 80°C Cross section 5 μm thick were cut in a cryostat and applied onto microscopic cover glass slips subsequently incubated in sp cific staining olutions

The myofibrillar ATPase method (Padykula and Herman 1955) as modified by Guth and Samaha (1969) was used for muscle fibre classi fication The reactions were carried out at pH 9 4 following alkaline pre incubation (pH 10 3) Using this procedure two distinct fibre typ s *can be distinguished one staining dark and the other with* a light staining intensity In the present study these two main fibre typ are referred to as fast twitch (FT) and slow twitch (ST) muscle fibre types respectiv ly (cf Gollnick t al 1972) The number of fibres of each type w s co nted from photomicrograph of th stain d cross sectio and the relative distribution f FT and ST fibres was determined A sample was discarded whenever the number f fibre failed to e ce d 200 Determinations of the percent of FT fibre i d plicat biopsies from the same muscle yielded a C V of 10% (n 10) The biop ies w re obtained at a d pth of 3 4 cm i to the muscle belly This standardization of sampling depth was not especially important since the distrib ti of FT and ST fibres appears homogeneous throughout the depth of the muscle (Edgerto et al 1975)

Fibre reas were determined from erial cross s ctions of each biopsy tained for NADH diaphorase (Novikoff et l 1961) This histochemical procedure was used to avoid fibre hrink ge du to dehydr tion 10 fibres of ach type (identified with th ATPase tai ing) were selected from an rea of the photomi rogr ph where the ross section appeared perpendi ular to the fibre orie tatio Pla imetry was sed to calculat the mean FT and ST reas in study I a d VI (cf Gollnick et al 1972) In study II a simpler method was sed in which the prints of the fibres were cut o t pooled tog ther a d w ighed By weighing of photopaper with k own areas an averaged area could b calculated for each fibre type Close agreeme t was found between th two methods and the method logical erro was calculated at 4% (C V)

Fibre area determinations on biopsies obtained from the same muscle on two different occasions revealed a C V of 16 17% for the absolute areas of FT and ST fibres respectively When the FT/ST area ratio was considered the corresponding figure was 9% The great variation in absolute values might have been due to a difference in degree of contraction caused by the sampling procedure The FT/ST area ratio was concluded to be a more re liable measure of muscle fibre area relationships This quotient is also included along with the relative number of fibres in the computation of relative muscle fibre area (Gollnick et al 1972)

Biochemical methods (IV VI)

Muscle specimens for enzyme activity analyses were trimmed quickly froze in liquid nitrogen and stored in a freezer at 80°C On the day of analysis the muscle samples were weighed in a cryostat (Karlsson 1971) and homogenized by sonication in 0 M KCl The homogenate was further diluted in buffer before 20 µl aliquots were added to 1 ml of test medium

The enzyme activity determinations were performed with fluorometric techniques using a Farrand ratio fluorometer-2 (Farrand Optical Co New York) The reactions catalyzed by the enzymes under investigation were coupled to NAD/NADP linked reac tions and the changes in NADH or NADPH were monitored according to principles outlined by Lowry (Lowry and Passonneau 1972) The initial rate of these changes was taken as the measure of enzyme activity The measurements were made under V_{max} conditions i e with an excess of substrate(s) By running standard curves with known amounts of fluorescence the experimental readings could be quantified The three major enzymes investigated in the present study were Mg^2 stimulated ATPase (E C 3 6 1 4) creatine phospho kinase (CPK E C 2 7 3 2) and myokinase (MK E C 2 7 4 3) These three enzymes will henceforth be referred to as contractility enzymes The composition of the test media and the methodological errors estimated from duplicate determinations made using the same homogenate are listed in Table II

Tabl II Summary of the reage ts used for enzyme activity determinations

E zyme	Reagent	C V [3]
Mg^{2} stimul t d ATPase [1]	0 05 M Tris (pH 8 1) 0 2 mM ATP 0 5 mM P enol pyruvate 0 015 mM NADH 0 02 % BSA 50 mM KCl 3 mM $MgCl_2$ auxiliary enzymes HLDH (0 54 U/ml pyruvate kinase (0 20 U/ml)	8 %
Myoki as [1]	0 1 M Tris (pH 8 0) 0 2 mM ADP 2 0 mM glucose 0 1 mM NADP 0 02 % BSA 5 mM $MgCl_2$ 1 0 mM glutathion auxiliary enzymes hexokinase (0 70 U/ml) gl cose 6 P dehydrogenase (0 14 U/ml)	4 %
Creatine phosphoki ase [2]	0 1 M tri etha olaminbuffer (pH 7 0) 35 mM creatine phosphate 1 0 mM ADP 20 mM gl cose 0 6 mM NADP 10 mM AMP 10 mM Mg acetate 9 0 mM glutathion auxiliary enzymes hexokinase ($\geq$ 1 2 U/ml) glucose 6 P dehydr genase ($\geq$ 1 2 U/ml)	4 %

[1] Adopted from Lowry Biochemicals were obtained from Sigma Chem Co St Louis USA and enzymes from Boehringer Mannh im GMBH W Germany except for HLDH that came from Worthington Biochem Corp N J USA

[2] CPK activated MONOTEST (cat No 15873) Boehringer Mannheim GMBH W Germany Before use the reagent w s diluted 1 10 in 0 1 M Tris buffer (pH 7 5)

[3] Coefficie t of varia ce calculat d from duplicate determinations on the same muscle homogenate

RESULTS AND COMMENTS

Force-velocity relationships in the knee extensor muscles (I II III)

The isokinetic muscle strength measurements made possible a comparison of maximal muscle force output both with respect to the position in the arc of motion and to different velocities of movement

With few exceptions individual peak torque values occurred at knee angles between 50 and 70 degrees (cf Fig ?) This knee angle tended to be smaller with increasing angular velocity due at least in part to the design of the apparatus (see Methodological aspects) The position for peak torque output was in close agreement with values previously reported for knee extension (Williams and Stutman 1959 Moffroid et al 1969) and reflects optimal relationships between muscle length and leverage at the knee joint

At each of the selected knee angles the torque produced was higher for isometric than for dynamic contractions With increasing angular velocities the torque prod ced decreased This was true throughout the range of motion although less pronou ced at the small knee angles

For comparative analyses the peak torque alues at each velo \ }y were selected resulting in torque velocity curves with the general shape of those in Fig 3 The peak isometric torque output ranged from 5 4 6 Nm/kg bw in a material comprising 5 physical education students The group mean of 3 45 Nm/kg bw was somewhat higher (n s) than the value obtained for an age matched group of sedentary men (mean 3 Nm/kg bw, n 10) Among the elite athletes the total range in peak isometric torque amo nted to 7 5 10 Nm/kg bw The values obtained by downhill skiers and sprinters/jumpers (3 9 and 3 8 Nm/kg bw) were significantly (p 0 05) higher than the averages of 3 1 achieved by the orienteers (cross country runners) Competition walkers did not differ significantly from a y of the other categories with respect to peak isometric torque (mea 3 4 Nm/kg bw) The prese t values are in accordance with values of about 3 0 Nm/kg bw reported for college men by Williams and Stutman (1959)

At the highest motion velocity studied i e 180 degrees/s the peak torque output ranged from 1 13 to 3 20 Nm/kg bw (n 74) The mean value obtained in the group of sprinters and jumpers (2 73 Nm/kg bw) was significantly (p < 0 01) higher than in any other group of subjects whereas the mean values for endurance athletes and physical education students did not differ signifi cantly from the 1 9 Nm/kg bw value for sedentary men

An average of about 55% of the maximal isometric torque re mained at 180 degrees/s Consequently the highest angular velocity offered by the apparatus was far from the maximal motion velocity which is defined as the maximal speed that can be achieved with 0 load (see Hill 1938) The estimated maximal knee extension velocity ranged 600 825 degrees/s (n 18) Thus the highest applied iso kinetic velocity corresponded to less than 30% of maximal motion velocity (Fig 3) Extrapolation of the torque velocity curve to the approximated maximal velocity values resulted in curves (Fig 3) similar to those previously reported by e g Wilkie (1950) and Asmus en et al (1965)

Forc velocity relationships and muscl fibre composition (I II III)

As indicated by the larg ranges in the torque produced at the diff rent velocities there w re inter individual differences in torq el city curves The values shown in Fig 3 suggest that muscle fibre c mposition might b o a factor determining the indi idual shape of the torque velocity c rve Although the groups of s bjects investigated were small the figure illustrates some ote worthy tenden ies Thus at 0 velo ity i e isometric c ntraction no differe c could be obtained between individuals with high and low perce tages of FT fibres This lack of correlation between fibre compo ition and maximal voluntary isometric stre gth also persisted i the total number of subj cts as well as when two leg isometric strength was considered (VI) This fi ding indicates that both FT nd ST motor units re involved in i ometric force development as s ggested by e g Gydikov a d Ko arov (1973) a d/or that the isometric force produced is eq al for both fibre types (cf Close 1972) Thus the q tity of muscle (n t meas red h re) and not the qu lity of muscle in terms of muscle fibre related properties could be d cisive to maximal shortlasti g isometric strength performance (cf Ikai and Fuk naga 1968)

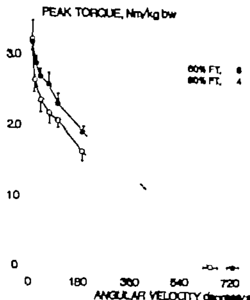


Fig re 3 P ak t rque lues pre d Nm pe kg f b dy weight ve g la vel ity i d grees p f t gro p f subj ts diff i g i fibre mpositi Th t rq vel ity c rve were t pol t d t the appro imat d l f ma imal velocity f k ten i V l es re me SE

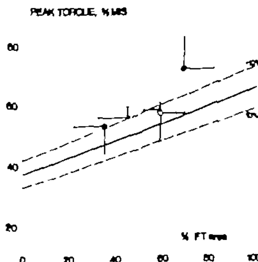


Fig re 4 P t rque xpres ed as perc t f ma imal i met i trength vs th rel tive are f fast t itch fibre Th regres i li (y 0 29 38 0 46 p < 0 05) wa d awn from i di idual lues f 2 h bit lly tive me (arg 33 60% FT are) d t pol t d t the treme po t Al includ d re me 1 (E) f lit thl t i t i g (O 7) ompetition w lki g (* 7) down ill kil g (A 8) ti g nd jumpi g (● 9) d g et d group of de t ry me (O 10)

Considering the other extreme point of the concentric force velocity curve i e maximal velocity a difference due to muscle fibre composition was indicated (Fig 3) Higher velocities were attained by individuals with a high percentage of FT fibres The correlation found between the percent of FT and maximal velocity was subsequently supported in similar analyses using the elapot equipment (Selapot Selcom AB Mölndal Sweden) (unpubl results from this department) These results are in accordance with findings in animals (see Burke and Edgerton 1975) and earlier indirect evidence from human sk letal muscle (Buchthal and Schmalbruch 1970)

The curves in Fig 3 also point to a difference in peak torque output during dynamic contractions depending on muscle fibre compo sition However this diff rence was less consistent Peak torque output could only be related to fibre composition at the highest velocity applied (180 degrees/s) Thus in a fairly homogenous material comprising 10 habitu lly active subjects a positive correlation (r 0 73 $p < 0.05$) was demo strated between peak torque at 180 degrees/s and the p rcent of FT fibres (Fig 5) Tendencie towards similar rel tion hips were also present in the more heterogeno s gro ps particip ting in studies I and II

Howev r when pe k torque o tput at the highe t velocity was e press d in relation to the maximal i ometric strength (MI) of each subject signifi ant positive correlation was obtained be twee this valu a d the proportio of FT muscle fibres (Fig 4) This relatio ship w s also s pported by the experiments o the different groups of athletes (Fig 4) Although the linear corre lation c efficients were relatively low the aggregate results underline the importa ce of a high proportio of FT mu cle fibres to force output at fast motion sp d Th s the high relative area of FT fibres in the elite athlete of sprinting and jumpi g would be adva tageous for their specific performance with respect to force output

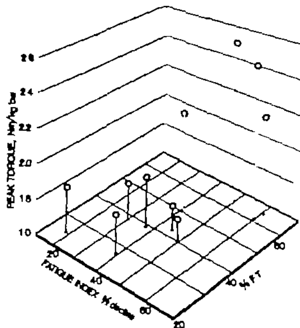


Fig re 5 Three dimensi l di gram howing i t rrel ti nships b tween th perc t g f f t twitch f bre (% FT) th p rc t g de li i p k t rque ft r 50 ep t d ma imal tra tio t 180 degre / (f tig i de) d peak torq e i Nm p kg of body ight (Nm/kg bw) produced i gl ont a ti ns t 180 d gre / for 10 habit lly tive me

Fatiguability and muscle fibre composition (III)

Animal experiments have shown that repetitive stimulation of muscles containing mainly FT fibres causes a rapid decline in force produced In the ST co nterparts on the other hand tension output is maintained over long periods of time This has been shown to apply to both single twitches and brief tetani (for refs see Burke 1973)

From the above it seems reasonable to assume that FT motor units are responsible for the major part of muscle force production during fast voluntary maximal contractions Repetitions of this kind of contraction could consequently provide some indication as to the interrelationship between fatiguability and muscle fibre types in human skeletal muscle

Thus experiments were performed on 10 habitually active men Their peak torque values displayed a gradual decline from about the 5th to the 45th contraction; values thereafter then reached a fairly stable plateau even up to 100 contractions However the magn tude of the decrease in peak torque varied between individuals The decline after 50 repetitions called the fatigue index demonstrated a positive correlation (r 0 86 p < 0 01) with the percentage of FT fibres (Fig 5) Thus a high proportion of FT fibres in the muscle appears to limit the ability to maintain maximal force at a high speed of motion This could be a factor to consider when evaluating the range in fibre compositions found among the elite athletes in sprinting and jumping events (II)

Contractility enzymes and muscle fibre types (V VI)

Our approach in the study of the contractile mechanisms in human mu cle was to examine the Mg^{2} timulated ATPase activity of the muscle A correlation between high activity for Mg^{2} stimulated ATPase and a high percentag of FT fibres in the investigated muscle biopsy homogenates of 14 subjects was not found until after a 8 week period of strength training The explanation for this finding is not known at present In experiments on pooled single muscle fibres a higher activity of Mg stimulated ATPase was found in FT fibre (Fig 6) The FT/ST ratio was pproximately 3 1 In imal skeletal muscle there is a higher acti ity for actomyosin ATP se i FT fibres (e g Barnard et al 1971 Guth and Samah 1969) and a similar relationship exists in human skeletal muscle for the ATPase activity of purified myosin (Taylor et al 1974)

Th activitie of CPK d MK were determined to assess the capacity of the mus le to replenish ATP rapidly No correlation was found betwee CPK activity nd fibre compositio in mixed muscle homogenates Thi was i conf rmity with previous fi dings for human skelet l muscle (Gollnick et al 1974) b t contrasted with the reports of Sherwi et al (1967 and 1969) o guinea pig and huma skeletal muscle indicating a signifi a tly higher CPK activity i FT than in ST muscles Reprod cing the CPK activity d termi ations o pool d FT and ST muscle fibres disclosed a 30%

greater activity in FT fibres a difference which could escape detection in the analysis of muscle homogenates with only a rather limited range of muscle fibre compositions

On the other hand a muscle fibre dependency could be observed for MK activity both by examination of mixed muscle homogenates and pooled single muscle fibres showing higher values in FT muscle fibres This finding has subsequently been confirmed by results on biopsies obtained using a larger number of subjects (Thorstensson to be published)

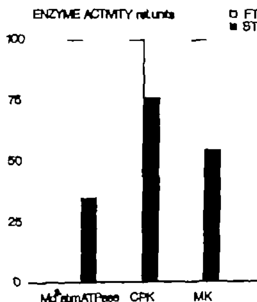


Fig re 6 E zyme ti itie f Mg^{2} timul t d ATP ti phosphoki (CPK) d my ki (MK) i po led i gl FT nd ST mu le fibre di s t d out from fre ze d ied bi psy mat ri l Th ti ity f ach nzyme i FT fibres se rbit rily t t 100

Isozymes of contractility enzymes and muscle fibre types (IV V)

The classification of human skeletal muscle fibres as FT or ST is based upon differenconces in pH lability between different forms of myofibrillar ATPase Several investigators have attributed these differences to the occurrence of two different isozymes of the myofibrillar ATPase (cf e g Trayer and Perry 1966 Sreter et al 1966 Lowey and Risby 1971) Howev r the techniques of protein separation used in the present study did not permit the conclusive demonstration of this difference in my fibrillar ATP se isozymes in human muscle fibre types

Two bands could be d monstrated that possessed CPK activity after isoelectric focusing of crude muscle homogenates the pI value averaged 7 2 and 6 9 respectively Comparison of isozyme staining de sity disclo d no difference in isozyme pattern b tween muscle samples from heart soleus or vastus laterali muscle The most alkali e ba d lways predominated constit ti g about 70% of total stain density Also when e amination of vastus lat ralis biopsy sp ime f diff re t fibre compositions was perf rmed no indications of a y fibr specificity in CPK iso yme p ttern were present These observations were c firmed by similar e perim nts o pooled single FT a d ST muscle fibres The results w re con iste t with the fi di g of two CPK isozyme t ntatively ide ti fied a th MM nd MB forms in huma heart a d skeletal muscle (Dawso and Fi e 1967 Somer and Konttinen 1972) Howe er other authors (e g Roberts et l 1974) hav reported th e istence of o ly o e CPK i ozyme form (th MM form) in h man skelet l mus le Methodologic l diff re ces might acco nt for these discrepanci s The muscle fibre specificity in CPK isozyme pattern fo nd for rabbit sk letal m scle (Sherwin et l 1967) was not confirmed by the pre e t re lts from human muscl

I the c f MK two isozyme b nds co ld be demonstrated after isoelectric foc ing Both were in the alk li e region of the gel pI l es averag d 9 6 (MK 1) and 8 9 (MK 2) respectively MK 1 w s the predomi ate i zyme irrespecti e of the source of muscl sampli g Th relative portio of MK 2 varied howe er and

attained higher values in the vastus lateralis than in soleus and heart muscle These findings are in close agreement with those obtained by Russel et al (1974) for human tissues using similar techniques They even suggested a heart and a skeletal muscle specific MK isozyme When the relative proportion of MK 2 in vastus lateralis muscle samples was related to the actual variation in muscle fibre composition a positive correlation was observed with respect to the percentage of FT fibres (Fig 7) The indicated fibre specificity in MK isozyme pattern was substantiated in experiments on pooled single FT and ST muscle fibres (Fig 7) The isozyme MK 2 made up 27% of total stain density in the FT fibres and 13% in the ST fibres respectively

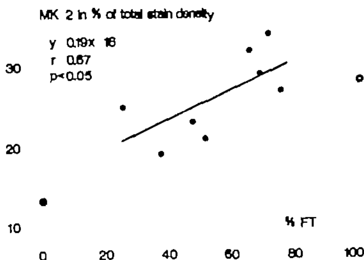


Fig re 7 Th tal density f my kinas 2 (MK 2) rel tive t total st d ty vs th perc t ge of FT fibres i 13 indi idual b op i I lud d re the l btal d f pool d i gl FT (◯) d ST (⊕) mus l fibres di t d t from fre d ied b psy mat i l

Effects of strength training (II VI)

The 8 week period of systematic progressive strength training aused significant ($p < 0.01$) improvements in isometric nd dynamic strength The relative increases in the various performance tests ranged from 13 87%

The percentage distribution of FT and ST muscle fibres was not altered by 8 we ks of strength training This is in accordance with other training studies on animals (for refs see Edgerton 1978) and human beings (Gollnick et al 1973 see also Prince et al 1976) The results support the view that fibre distribution based on myo fibrillar ATPa i gov rned by largely genetic factors (Komi et l 1976)

However the relative volume of fibre types in a muscle can still be ltered by a change in the FT/ST area ratios This ratio was increased by strength training (Fig 8) indic ting specific hypertrophy of FT fibres due to the tr ining stimuli Similar re sults were obtained aft r an tt mpt with strength training of primates (Edg rton 1976) Thu the high FT/ST area ratios found in sprinters nd jump rs (Fig 8) may be the result of a le gthy period f strength training Similar res lts were obtained in weight lifters (Edström nd Ekblom 1972 Pri ce et al 1976)

The effect of strength training o th activity of the selected zymes w s insignificant e cept in the case of MK for which a small i crease was obs rved In a later study involving the ame type of stre gth traini g o significant changes in the activitie of any of the co tr otility e zymes were obt ined (Thorstensson et al 1976) How ver the cha ged V_{ma} f the enzymes does not exclude the possibility of ch ges in the kinetic properties or isozyme patterns Studies are ow in progress to evaluat this possibility The e act nature of the stre gth trai i g progr mme also appe rs t i fl the eff ct on the zyme a tivities This was indi ted by the increased activity ob erved for CPK and MK after a strength traini g programme carried out a sprint training (Th rste sson et al 1975) It is lik ly that higher dema ds re placed n r pid ATP ynthesis u der co ditions i which fast ma imal co tractions are repeated 5 8 times with brief rest i tervals as i sprint trai i g

attained higher values in the vastus lateralis than in soleus and heart muscle These findings are in close agreement with those obtained by Russel et al (1974) for human tissues using similar techniques They even suggested a heart and a skeletal muscle specific MK isozyme When the relative proportion of MK 2 in vastus lateralis muscle samples was related to the actual variation in muscle fibre composition a positive correlation was observed with respect to the percentage of FT fibres (Fig 7) The indicated fibre specificity in MK isozyme pattern was substantiated in experiments on pooled single FT and ST muscle fibres (Fig 7) The isozyme MK 2 made up 27% of total stain density in the FT fibres and 13% in the ST fibres respectively

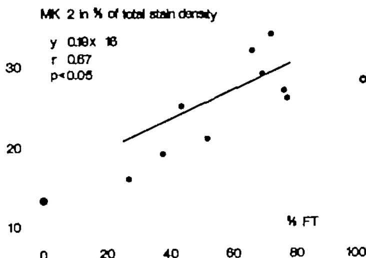


Fig re 7 Th t i d nsity f my ki 2 (MK 2) rel ti t tot l t i d ity vs th perc t g f FT fibres i 13 i d idual biopsi I luded re th l es bt i ed f pool d i gl FT (●) nd ST (⊕) mu l fibres di ect d t from fre d i d bi psy mat i l

GENERAL DISCUSSION

The present study has shown that force output and fatigue in voluntary contractions can be correlated to the proportion of FT muscle fibres on the one hand and that differences exist between FT and ST muscle fibres with respect to contractility nzymes and th ir isozymes on the other hand

The fact that high proportio of FT fibres is ess ntial to a high force output during fast muscle contractions may be d e to several diff rent circumstances The mo t likely caus is the short contraction time of FT fibres whi h is related to higher intrinsic speed of short ning a well a a higher rate of rise in tension in a tetanu (Close 1964) A higher rate of tensio d velopment should yield higher forc o tput at the optimal angle in th arc of motio in the present experiment Furth rmore a high r intrinsic speed f shorteni g in FT fibres hould if the shape of the forc vel city c rves are ro ghly similar lead to a divergence of the curves f r FT d ST fibre a d higher forc output for FT fibres t high co tr ction velo iti ev if there i o difference in maximal is metric forc b tw n fibre type (cf Fig 3)

It is co eivabl that the rat of hydrolysis of ATP at the cross bridg b tw n cti a d myo in in the muscle is o e of the mai factors trolling th pe d f h rt ni g and thus even the te io produ d at high ontr tion lociti s Thi ATP hydrolysis i bro ght bout by th e zymatic acti ity f cto myosi ATP e Th higher a tivity f this e zyme f und in FT fibre w uld therefore pro ide basis f r the diff re c s obtained i c ntractile properties O r i creased k owledg ab t the morph logy d bio hemistry of m s le excit tion- ontractio c pli g h i di at d oth r p ible ite of control u h s the release nd uptak f tr smitter s bsta ce in the ro musc l r j tio a well s of Ca iqns b tween the rcoplasmi reticulum d the co tractil app rat Differe s betw n FT nd ST mot r it i th re p ct hav al be d monstrat d (for ref B rk d Edgert 1975)

The potential of a faster hydrolysis of ATP in FT muscle fibres was matched by higher activity for the enzymes CPK and MK both serving to catalyze reactions providing rapid resynthesis of ATP The fact that both these pathways are open has been demonstrated during single contractions of frog muscle (Cain and Davies 1962) The extent to which they are decisive to the speed and force of contraction is unknown at present The presence of isozymes of both CPK and MK in the human muscle cell is interesting in this context Thus the MM form of CPK has been shown to bind specifically to the M band of the contractile apparatus (Turner et al 1973) Similar relationships for the MK isozymes have not been investigated although the last traces of MK have been very difficult to remove in preparations of myosin (Noda 1973)

The difference in MK activity between fibre types is also likely to be of significance in the regulation of the overall metabolism of the two kinds of muscle cells The ratio between the adenylate nucleotides involved in the MK reaction regulates the activity of several key enzymes in the energy yielding processes (cf Atkinson 1968) The effects of AMP on the glycolytic regulatory enzyme phosphofruktokinase has been emphasized in this respect (Newsholme 1971) The higher activity of MK in human FT muscle fibres also coincides with a higher anaerobic capacity of this hre type (e g Essén et al 1975 Sjödin 1976) Extended studies the kinetics and localization of MK and its isozymes are re ired in order to clarify their role in co nection with muscle contraction

The anaerobic metabolic profile of FT muscle fibres may explain for the greater fatiguability of this fibre type Repeated activation of FT motor units are likely to create local conditions in the FT muscle fibres such as a decrease in pH or available substrates detrimental to continued usage Differences in oxidative potential within the FT motor unit pool might affect the resistance to fatigue This appears to be the case in animal muscle in which two FT motor unit types can be identified The one with a high oxidative capacity is comparatively resistant to fatigue whereas the other displays a low oxidative potential and is easily fatigued (see Burke and Edgerto 1975) The extent to which s bgroups of human FT muscle fibres characterized by histochemical means

(cf Brooke and Kai er 1970 Edgerton et al 1975 Essén et al 1975) are affecting the susceptibility to fatigue and other contractile properties is at present unclear The possibility of a difference in fatiguability confined to other parts of the neuro muscular system u h as the properties of the axons or the neuro muscular junction cannot be excluded completely (see Burke 1973) Support for the latter possibility has been presented by Stephens and Taylor (1972) They concluded that neuromuscular junction fatigue w s mo t important during the initial ph e (about 1 min) of maintain d ma imal contractions whereas the fatigue confi ed to the contr ctile leme ts incr ased with l ng r contra tion times

The metabolic morphologic nd contractile properties of motor units app ar to be i timately rrelated with the p ttern of motor unit recruitme t during different movements It i gen rally accept d that mot r unit under most co ditions are activated o ording to size i e mall motoneurons prior to large one This ize principl w first p stul t d by H nneman t al (1965) i de erebrated cats a d has inc be n supp rted in studie on various animals including man (g Milner-Brow t al 1973) These investigatio w re perform d m inly using isometric contractions with rel tiv ly low te ions or dynami co tractions with a slow increa e in te si n o tp t How ver an in r asing amo nt f ide ce demonstrates th t the rd r of motor unit recruitment is not e tirely fixed (e review by Burke and Edg rton 1975) The ce tral rvou syst m appears t be abl to adju t th s qu nce f motor nit re ruitment to sel ctively involve large units (presumably FT) when very forceful nd/ r r pid moveme ts are req ired (cf Grimby and H er 1968 Warmolts and Engel 1972) Th fa t ma imal is ki ti co tra tio s used in the pre ent tudy ppear to be good exampl of h mo eme ts F rthermore if it is accepted th t th great t hypertrophy oc urs in muscl fibr with the gre t st i re i sage the th prese t results comprising an i re e i th FT/ST are ratio with strength trai i g s well a high FT/ST area ratio v lue in athletes i explosive e t wo ld l d upport to the view that relati e invol eme t of diff re t motor nit types c n be changed d ri g movement lli g for b th great force and speed

SUMMARY

Force velocity relationships were identified by studying iso kinetic contractions of the knee extensor muscles of healthy male individuals of varying proficiency and varying degrees of strength training

No correlation was found between force output and muscle fibre composition except at the highest angular velocity applied A positive correlation between peak force and the percentage of fast twitch muscle fibres was noted at this v locity

The susceptibility to fatigue during repeated fast maximal voluntary contractions was shown to be related to muscle fibre composition; high fatiguability coincided with a high percentage of fast twitch fibres

Examination of elite athletes in explosive strength events disclosed a wide variation in the perc nt of fast twitch fibres but a significantly higher fast to slow twitch fibre area ratio than in sedentary subjects and endurance athletes

The fast to slow twitch fibre area ratio increased after a period of systematic stre gth training indicating a specific effect for the heavy training loads on the fast twitch muscle fibres ever the training effect on selected enzyme activities was ght irrespective of any large gains in strength performance

A alysis of mi ed muscle homogenates i dicated higher activity for Mg^{2} stimulated ATPase nd myoki ase in fast than in slow twitch muscle fibres This indicated fibre sp cificity was co firmed for both enzymes in a alysis of pooled single freeze dried mus l fibres

The distrib tio of isozymes of myokinase separated by is electric focusing was shown to be related to fibre type distri bution in muscle homogenates Similar results were obtained when applying this procedure to pooled single freeze dried muscle fibres A correspo ding fibre dependency could not be found for the distribution of isozymes of creatine phosphokinase

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SODIUM AND ANGIOTENSIN II IN THE CENTRAL CONTROL OF FLUID BALANCE

A study in conscious goats

BY

LEA ERIKSSON

HELSINKI 1976

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I Andersson B L Eriksson O Fernández C -G Kolmodin and R Oltner Centrally mediated effects of sodium and angiotensin II on arterial blood pressure and fluid balance Acta physiol scand 1972 85 398 407

II Eriksson L Effect of lowered CSF sodium concentration on the central control of fluid balance Acta physiol scand 1974 91 61-68

III Eriksson L Negligible role of CSF cations other than Na^+ in the central regulation of ADH release Acta physiol scand 1976 97 398-400

IV Eriksson L and F Fyhrquist Plasma renin activity following central infusion of angiotensin II and altered CSF sodium concentration in the conscious goat Acta physiol scand 1976 98 209 216

V Eriksson L and F Fyhrquist A comparison between the central effects of angiotensin II and its fragments des_1-angiotensin II and $des_{1\ 2}$-angiotensin II Acta physiol scand 1976 96 134-136

These articles will be referred to by their Roman numerals

Abbreviations used

ADH = antidiuretic hormone

C_{H_2O} = renal free water clearance

CNS = central nervous system

CSF = cerebrospinal fluid

ECF = extracellular fluid

ICV = intracerebroventricular

PRA = plasma renin activity

SFO = subfornical organ

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INTRODUCTION

Verney s classical experiments (1947) in unanesthetized dogs form the basis for the theory of an osmometric regulation of the antidiuretic hormone (ADH) release He found that infusions of hypertonic solutions of sodium salts or sucrose into the carotid artery caused antidiuresis in hydrated dogs whereas hypertonic urea was ineffective Verney postulated that there are osmoreceptors in the brain probably in the anterior hypothalamus which regulate the release of ADH (Verney 1947 Jewell and Verney 1957) He inferred that the adequate stimulus for the osmoreceptors is not a rise in blood tonicity as such but rather changes in the composition of the extracellular fluid (ECF) which by dehydration reduce the volume of the receptors This would explain why an elevation of ECF osmolality induced by a highly diffusible substance like urea did not stimulate the ADH release ADH release and drinking serve a common purpose i e they maintain the water balance When Andersson (1953) found that injections of small amounts of hypertonic NaCl solution into the anterio-medial hypothalamus elicited drinking in the goat he postulated that cerebral osmoreceptors also participate in the regulation of water intake

The possible influence of brain barrier systems was not taken into consideration in these original investigations Latter studies in the conscious goat suggest that changes in cerebrospinal fluid (CSF) sodium concentration are much more effective in influencing the central control of fluid balance than are changes in CSF osmolality Therefore a possible alternative to osmoreceptors in Verney s sense would be sodium sensitive receptors near the third cerebral ventricle (cf Andersson 1971) Here the concept of specific cerebral sodium sensitivity will be discussed in relation to the results of the present experimental studies

The physiological importance of the renin angiotensin system had originally been related only to the peripheral effects of angiotensin II (vasoconstriction stimulation of aldosterone secretion) However in 1961 Bickerton and Buckley demonstrated that angiotensin II can produce a centrally mediated rise in

blood pressure Previous to this Linazasoro and co-workers (1954) had observed that injections of kidney extracts elicit drinking in rats The association between the renin-angiotensin system and the thirst mechanism has been further studied by Fitzsimons and co-workers (cf Fitzsimons 1972) The effects of systemically administered angiotensin II and hypertonic NaCl solution are additive in the rat (Fitzsimons and Simons 1969) The dipsogenic effect of angiotensin is centrally mediated Ad ministration of intravenously inactive amounts of this peptide into the anterior hypothalamus in rats (Epstein Fitzsimons and Simons 1969) or into the third ventricle in goats (Andersson and Westbye 1970) elicits drinking Furthermore angiotensin II stimulates the release of ADH from the neurohypophysis (Severs et al 1970 Bonjour and Malvin 1970) Even this effect is due to an action of the octapeptide at the hypothalamic level of the brain (Andersson and Westbye 1970)

Studies on the centrally mediated effects of angiotensin have given interesting results in recent years (for review see Severs and Daniels-Severs 1973) Particularly the discovery of an intrinsic renin-angiotensin system in the brain (Ganten et al 1971 Fischer-Ferraro et al 1971) supports the contention that angiotensin II may be physiologically important within the central nervous system (CNS)

Electrical stimulation of different brain loci and intracerebroventricular (ICV) perfusion with low $[Na^+]$ CSF in turn have furnished evidence that the CNS participates in the control of renin release from the kidney (for review see Davis and Freeman 1976) Davis and Freeman (1976) divide the mechanisms controlling renin release into following groups: the intrarenal receptors the sympathetic nerves and humoral agents Humoral regulators include sodium and potassium ions angiotensin II and ADH Angiotensin and ADH inhibit the secretion of renin proba bly by a direct action on the juxtaglomerular cells (see Davis and Freeman 1976) In addition to this short-loop negative feedback angiotensin II has been postulated to suppress the renal renin release by raising the blood pressure in part via central nervous action (Oparil and Haber 1974)

THE PURPOSE OF THE STUDY

'he principal aims of the experimental work in this thesis rere to study in conscious goats:

- the role of CSF sodium concentration in the control of fluid balance (papers II III and IV)

- the influence of ICV infusions of angiotensin II and of its fragments des$_1$-angiotensin II and des$_{1\ 2}$-angiotensin II on fluid balance (papers I IV and V)

- the importance of CSF sodium concentration for centrally mediated responses to angiotensin II (paper I)

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ressure transducer The mean and systolic/diastolic blood ressure and the heart rate were recorded on an ink-writing olygraph Entry of blood into the catheter and blood clotting luring testing was prevented by infusing isotonic NaCl solution lowly (0 1 ml/min) into the catheter system

Radioimmunoa say of PRA and plasma angiotensin II concentration
Blood samples were taken via a Braunule cannula from the jugular vein into prechilled centrifuge tubes with 0 3 M EDTA solution as an anticoagulant immediately cooled and centrifuged at +4°C The plasma was stored at -20°C until radioimmunoassayed

Radioimmunoassay of PRA (Fyhrquist et al 1976) was modified for the assay of goat PRA The optimal pH for angiotensin I generation in goat plasma was found to be 6 2 Therefore samples were incubated at pH 6 2 in the presence of OH-quinoline 3 5 mM and Na_2-EDTA 15 mM There was no change of pH during the 2 hour incubation at +37°C

Radioimmunoassay of plasma angiotensin II concentration was made according to Fyhrquist et al (1972)

Special reagents
The angiotensin analogues used were:
- angiotensin II (val^5-angiotensin II-asp-γ amide Hypertensin® Ciba)
- angiotensin III (des$_1$-ileu^5-angiotensin II Schwarz-Mann)
- des$_{1\ 2}$-ileu^5-angiotensin II (Schwarz-Mann)

RESULTS AND COMMENTS

1 Centrally mediated effects of angiotensin II and sodium on fluid balance and blood pressure (paper I)

When angiotensin II (0 8 or 0 4 ng kg^{-1} min^{-1}) was infused in hypertonic NaCl (0 25 or 0 33 M) solution into the third cerebral ventricle of hydrated goats for one hour conspicuous dipsogenic antidiuretic hypertensive and natriuretic responses were obtained If the CSF $[Na^{+}]$ was simultaneously lowered by infusing angiotensin dissolved in isotonic d-glucose the effects of the octapeptide were markedly reduced Angiotensin in 0 14 M NaCl solution or merely the hypertonic NaCl gave moderate responses In the following sections the different effects of the infusions are considered separately

Water intake

In order to avoid over-hydration hydrated goats were not allowed [illegible] more than 500 ml During the combined infusion of angio-[illegible]n and hypertonic NaCl all goats drank the total amount of [illegible]ter available The latency time for drinking was short and thirst outlasted the infusion period by up to 30 min Infusion of angiotensin in 0 14 M NaCl or of hypertonic NaCl alone induced drinking in most cases but the latency time was longer and drinking stopped within 10 min after the infusion None of the goats drank during the infusion of angiotensin in isotonic glucose but some goats sipped water <u>after</u> termination of the infusion In non-hydrated goats it is not necessary to withhold water In such animals simultaneous infusion of angiotensin and hypertonic NaCl results in a marked potentiation of the dipsogenic effect (Andersson and Westbye 1970) Andersson and co-workers (Andersson Leksell and Rundgren 1975) have recently shown that such potentiation also may be obtained when the infusion of angiotensin and hypertonic NaCl are not concurrent Angiotensin dissolved in 0 3 M glucose failed to elicit drinking but it markedly accentuated the dipsogenic and antidiuretic effects of the subsequent infusion of hypertonic NaCl This delayed effect of angiotensin would suggest the possibility that angiotensin remains at periventricular receptor sites and continues to interact with Na^{+} thereby eliciting thirst and ADH release for about

half an hour Another possibility is that a sustained angiotensin-induced receptor activation is implicated In the rat injections of angiotensin II dissolved in solutions with vari- ionic composition directly into the septal and preoptic areas of the brain have given partially divergent responses (Swanson Sharpe and Griffin 1973) Hypertonic NaCl mixed with threshold doses of angiotensin was found to potentiate drink- i On the other hand there was no decrease in the dipsogenic effect when angiotensin was injected in Na^+-free solution)n hypertonic KCl was infused with angiotensin a marked reduction of water intake was seen

Inhibition of water diuresis

A strong and long lasting antidiuretic effect was obtained by the infusion of angiotensin II in hypertonic NaCl The antidiuresis seen in response to the infusion of angiotensin in 0 14 NaCl was of shorter duration No antidiuretic response or only a weak one was observed during the ICV infusion of angiotensin in isotonic glucose However a distinct post-infusion inhibition of the water diuresis was seen after the infusion .is delayed response may indicate that the receptors activated by angiotensin remain in the activated state which is unmasked when glucose no longer reduces the CSF $[Na^+]$

Evidence that the inhibition of the water diuresis elicited by these stimuli is actually due to release of ADH was first obtained indirectly It was shown that the ICV infusion of hypertonic NaCl (Andersson Dallman and Olsson 1969) and angiotensin II (paper I) no longer caused an antidiuresis when the hypothalamo-neurohypophyseal connections were interrupted by radiofrequency lesions in the median eminence Recently Lishajko and Andersson (1975) have bioassayed the ADH in urine following the infusion of angiotensin II and hypertonic NaCl into the ventricular system of the hydrated goat Plasma samples obtained during infusions in the later studies (papers IV and V) were analyzed for ADH with a sensitive direct radioimmunoassay method (Fyhrquist Eriksson and Wallenius 1976) Plasma ADH concentrations rapidly rose during infusions of angiotensin II and hypertonic NaCl

Olsson and Kolmodin (1974) have further studied the angiotensin-Na^+ synergism by giving these stimuli via different

routes Infusions of angiotensin II into the lateral cerebral ventricle in amounts which had negligible antidiuretic and no dipsogenic activity on their own potentiated the antidiuretic and dipsogenic effect of simultaneous intracarotid infusions of hypertonic NaCl in hydrated goats

Hypertensive and natriuretic effects

During the ICV infusion of angiotensin II in hypertonic NaCl the blood pressure began to rise within 5 min reaching its maximum increment (25-45 mmHg) within 20 min and remained elevated 50 to 90 min after cessation of the infusion Also with angiotensin in 0 14 M NaCl the rise of blood pressure was rapid (maximum increment 15-35 mmHg) but the pressure started to decline with the smaller doses before the infusion was completed The blood pressure rose slowly during the infusion of hypertonic NaCl alone reaching the highest increment (15-30 mmHg) at the end of the infusion period Infusion of angiotensin in isotonic glucose had no noticeable effect on the blood pressure These experiments strongly suggest that an angiotensin-Na^+ sensitive receptor system may contribute to the central regulation of the arterial blood pressure

Bergmann et al (1971) have postulated that Na^+ may form a complex with angiotensin and thus a biologically more active molecule This could explain the Na^+-angiotensin synergism observed in the goat To reexamine this possibility angiotensin II was pre-incubated in saline before it was infused with glucose (paper I) The effect was not enhanced Furthermore the intravenous injection of angiotensin II in glucose caused the same rise in carotid blood pressure as when it was injected in isotonic NaCl

Although great variations were seen between animals the magnitude and the duration of the natriuretic response to the different infusions were generally correlated to the rise in blood pressure It has previously been shown that the natriuresis induced by hypertonic NaCl applied into CSF is accompanied by an increase in the glomerular filtration rate (Andersson Dallman and Olsson 1969) The correlation between the hypertensive and natriuretic effect in the present study also indicates that changes in renal hemodynamics may have contributed to the natriuretic response

In the present study it was found that the local Na^+ concentration determines the degree to which angiotensin II applied in the third ventricle affects the fluid balance and elevates the blood pressure It is therefore suggested that angiotensin II may act *via* a Na^+-sensitive receptor system near the third ventricle Angiotensin II may either make the receptors more sensitive to the environmental Na^+ concentration or facilitate the entry of Na^+ to the receptor sites

Recordings of action potentials in different brain loci have shown that angiotensin II has a direct effect on many central neurons (Nicoll and Barker 1971 Wayner Ono and Nolley 1973 and Felix and Akert 1974) Furthermore Wayner et al (1973) have found that some of the Na^+-sensitive neurons which all were sensitive to angiotensin II displayed a pronounced potentiation of discharge frequencies when both stimuli were applied simultaneously They suggest that angiotensin facilitates membrane Na^+ permeability Angiotensin II is known to affect toad bladder sodium flux (McAfee and Locke 1967) and intestinal electrolyte transport (e g Davies Munday and Parsons 1970) The study of Gutman et al (1972) gives good evidence that angiotensin stimulates Na^+ transport in the tissues where it is acting They found that angiotensin II increases microsomal (Na^+-K^+)-ATPase activity in the hypothalamus the mucosa of colon and the adrenal cortex

2 Effects of lowered CSF Na^+ concentration on the central control of fluid balance (papers II and III)

Water diuresis

The concentration of CSF $[Na^+]$ was artificially lowered by infusing isotonic or hypertonic non-electrolyte solutions into the cerebral ventricular system of goats in normal water balance Either the third ventricle was infused for 40 min at 10 or 20 µl/min or the lateral ventricle was infused for one hour at 20 µl/min All isotonic non-electrolyte solutions caused water diuresis and so did hypertonic (0 45 or 0 6 M) solutions of carbohydrates Hence the increase in renal free water clearance (C_{H_2O}) was not caused by reduced osmolality of CSF

more likely by dilution of the CSF $[Na^+]$ These infusions obviously inhibit the release of ADH normally occuring in the non-hydrated animal In the first place there was no increase in renal osmotic clearance Secondly intravenous injection of 5 mU of ADH interrupted this water diuresis

The infusions of non-electrolyte solutions into the ventricular system also reduced the relative concentrations of other ions in the CSF to the same degree as the Na^+ concentration Therefore it could be argued that the diuretic effect of these infusions may have been due to a reduced concentration of some ion or solute other than Na^+ Control infusions however and previous studies in the same species seem to rule out this possibility Infusions of isotonic saline alone or saccharides in isotonic saline which maintain the CSF $[Na^+]$ but reduce the concentrations of other ions did not produce water diuresis However after the combined infusions some increase in C_{H_2O} was often seen Osmotically these hypertonic solutions may have drawn water into the CSF thereby reducing the Na^+ concentration and making a postinfusion reduction of the ADH release apparent On the basis of earlier studies in the goat (Andersson et al 1967) it is unlikely that reduced CSF Cl^- concentration was of crucial importance

In order to reduce the CSF $[Na^+]$ without reducing the normal concentration of other cations isotonic saccharides with K^+ (3 mmol/l) Ca^{2+} (1 3 mmol/l) and Mg^{2+} (1 mmol/l) were infused into the ventricular system (paper III) These infusions caused water diuresis on most occasions However when the cat ions were infused together with d-glucose the renal C_{H_2O} sometimes failed to turn positive It was found that glucose with Ca^{2+} and Mg^{2+} consistently induced water diuresis while glucose with K^+ did not always do so This latter result with K^+ and glucose does not disprove the crucial role of Na^+ It is possible that glucose with K^+ in some way activated the Na^+ transport in periventricular tissues and choroid plexuses Brøndsted (1970) has studied the transport of various sugars from CSF He found a carrier-mediated transport of glucose which seems to be connected with the active transport of Na^+ and K^+ The net fluxes of Na^+ and K^+ showed that if one molecule of K^+ passes from the perfusate into surrounding tissues 3-10 molecules of Na^+ would

pass in the opposite direction Recent studies in goats have given additional information concerning the periventricular sodium receptors (Olsson 1976 Leksell Lishajko and Rundgren 1976) ICV infusions of isotonic NaCl dissolved in heavy water (D_2O) elicit in spite of initially sufficient CSF [Na^+] a pronounced water diuresis in the non-hydrated goat (Leksell et al 1976) Deuterated water is an inhibitor of (Na^+-K^+)-ATPase (Ahmed and Foster 1974) It appears possible that ICV infusions of deuterated water inhibit the release of ADH by affecting the Na^+ transporting enzyme system and so reducing the Na^+ sensitivity of the periventricular receptors Substances which stimulate this enzyme system could thus increase the Na^+ sensitivity This may be the situation with glucose together with K^+

In most of the studies reported perfusion of the cerebral ventricular system with artificial CSF low in Na^+ has a diuretic effect In chloralose-anesthetized dogs a brisk water diuresis ensues (Leusen and Lacroix 1961) In conscious sheep urine flow rate shows a tendency to increase sometimes markedly (Mouw et al 1974) On the other hand pentobarbital-anesthetized dogs have a high initial plasma ADH concentration which does not show any consistent change during low [Na^+] CSF perfusion (Mouw and Vander 1971) It is known that several anesthetics increase the secretion of the ADH (cf Walker 1957)

Renal Na^+ excretion

Artificial lowering of CSF [Na^+] exerts varying effects on the renal electrolyte excretion In pentobarbital-anesthetized dogs (Mouw and Vander 1970) and in conscious sheep (Mouw et al 1974) urine Na^+ excretion decreased during ventricular perfusion with low [Na^+] CSF However when a water diuresis developed as in the present study in chloralose-anesthetized dogs (Leusen and Lacroix 1961) and occasionally in the sheep (Mouw et al 1974) there was no consistent antinatriuresis This dissimilarity of responses raises the question of a possible interrelationship between the renal sodium and the renal water excretion In order to keep urine flow stable while lowering the CSF [Na^+] isotonic fructose was also infused into the CSF in hydrated goats (paper IV) Na^+ excretion did not decrease Thus an increase in urine flow could not explain the lack of antinatriuretic response to the ICV fructose infusion in non-hydrated goats On the other

hand there was some decrease in the renal K^+ excretion

3 Effect on PRA of ICV infusions of angiotensin II and altered CSF Na^+ concentration (paper IV)

Angiotensin II

Infusion of angiotensin II dissolved in 0 15 M NaCl in doses from 0 5 to 1 µg into the lateral cerebral ventricle caused a marked decrease of PRA The direct inhibitory effect of angiotensin on the renal renin release (cf Davis and Freeman 1976) was excluded in these experiments since the plasma angiotensin II concentration did not rise after the infusions However the infusions also raised arterial blood pressure (25-30 mmHg) released ADH and increased renal Na^+ excretion Any one of these effects may have influenced renin release

Centrally applied angiotensin II is thought to increase the sympathetic outflow (cf Severs and Daniels-Severs 1973) In view of this Vollmer et al (1974) have suggested that an angiotensin II-induced increase in adrenergic outflow may stimulate the renal renin production On the other hand Oparil and Haber (1974) have assumed that the centrally mediated pressor effect of angiotensin may be of considerable importance in the negative feedback of angiotensin on renin release The present study indicates that angiotensin II applied into the CSF suppresses PRA but does not unmask the underlying mechanism

Hypertonic NaCl

Like the infusions of angiotensin II infusion of 0 5 M NaCl solution at a rate of 20 µl/min for 30 min caused a definite reduction of PRA As expected from earlier studies in this species (Andersson et al 1967) these infusions also caused antidiuresis natriuresis and some drinking Blood pressure response was variable In some experiments (n = 2) there was no appreciable change in blood pressure whereas in other recordings (n = 2) blood pressure rose slowly and reached the highest increment (10-15 mmHg) at the end of the infusion At that time PRA was already suppressed Because of the rather long half-life of renin in circulating blood (10-20 min) it seems unlikely that the rise

in blood pressure could be of importance to the PRA suppression in these experiments On the other hand the infusion of 0 5 M NaCl caused a large rapid increase in plasma ADH concentration (Fyhrquist Eriksson and Wallenius 1976) ADH is known to inhibit renin release from the kidney (see Davis and Freeman 1976) It therefore appears probable that the increasing of CSF $[Na^+]$ suppressed PRA by stimulating ADH release

In conscious rats Morris Campbell and Pettinger (1976) have recently reported results similar to our data Injection of hypertonic (2 M) NaCl into the third ventricle of the rat caused a decrease in serum renin activity a rise in blood pressure and natriuresis In contrast high $[Na^+]$ CSF perfusion of the ventricular system in Na^+-depleted conscious sheep influenced neither blood pressure nor plasma renin concentration (Abraham et al 1976) The varying Na^+ balance may readily explain the apparently diverging results The hyperactive state of the renin-angiotensin system following Na^+-depletion in the sheep may not be suitable for the demonstration of renin suppression with hypertonic NaCl applied into the cerebral ventricle Hyponatremia and hypovolemia may be too effective peripheral stimuli for renin release to be suppressed by a rise in CSF $[Na^+]$

Lowered CSF Na^+ concentration

Lowering of CSF $[Na^+]$ by infusing isotonic fructose into the lateral cerebral ventricle at a rate of 20 µl/min during one hour elevated PRA The result agrees with the studies of low $[Na^+]$ CSF perfusion in pentobarbital-anesthetized dogs (Mouw and Vander 1970) and in conscious sheep (Mouw et al 1974) In the non-hydrated goats the infusion of fructose induced water diuresis without any consistent change in renal Na^+ excretion in agreement with earlier studies (paper II) In the hydrated goats the urine flow rate was stable but yet there was no obvious reduction of renal Na^+ excretion following the infusion of fructose However both in hydrated and non-hydrated goats renal K^+ excretion decreased somewhat

Infusion of isotonic NaCl into the lateral ventricle during one hour caused some increase in PRA This result cannot be explained at present It should be stressed however that the

rise in PRA after the infusion of saline was small (35 %) compared with that following the infusion of fructose (155 % in hydrated and 125 % in non-hydrated goats)

The efferent pathway by which the lowered CSF $[Na^+]$ influences the juxtaglomerular apparatus remains unclear The blood pressure was stable during the infusion (paper II) Thus the renal baroreceptor mechanism would appear not to be responsible for the change in PRA The decrease in plasma ADH could allow for a rise in PRA However in hydrated goats too PRA rose in spite of initially suppressed ADH Furthermore in pentobarbital-anesthetized dogs Mouw and Vander (1971) found an increase in PRA even though the plasma ADH was high and did not show any consistent changes In our study there were no changes in renal Na^+ excretion which would explain the stimulation of renin release The role of decreasing renal K^+ excretion due to infusion remains to be settled Mouw and Vander (1971) showed that the renal nerves are not necessary for the antinatriuretic effect of low $[Na^+]$ CSF perfusion On the other hand their study did not exclude the renal nerves as one possible pathway to the juxtaglomerular cells during lowering of CSF $[Na^+]$ In anesthetized cats Schad and Seller (1975) reported that high $[Na^+]$ CSF perfusion of the ventricular system produced an increase in renal sympathetic activity whereas a low $[Na^+]$ perfusion decreased it It would appear necessary to evaluate whether CSF $[Na^+]$ can have an effect on the juxtaglomerular cells <u>via</u> the renal nerves

4 A comparison between the centrally mediated effects of angiotensin II and its fragments des_1-angiotensin II and $des_{1\ 2}$-angiotensin II (paper V)

As expected from previous studies in the hydrated goat (paper I) infusion of angiotensin II in 0 15 M NaCl into the lateral ventricle at a rate of 50 ng/min during 30 min caused drinking antidiuresis and natriuresis In contrast infusion of des_1-angiotensin II (angiotensin III) at an equimolar concentration did not induce any drinking or antidiuresis and had no obvious influence on renal Na^+ excretion When a more concentrated angiotensin III solution was infused at a rate of 0 35 µg/min

during 5 min three out of six animals drank water There was some inhibition of water diuresis after every infusion and C_{H_2O} became negative in 5 out of 8 experiments These responses as well as the rise in blood pressure were definitely smaller than those following infusion of angiotensin II of half the molar strength Infusion of $des_{1\ 2}$-angiotensin II the hexapeptide had no effect on water balance

Fitzsimons (1971) studied the dipsogenic action of various angiotensin analogues He found that the des_1-angiotensin II retains about 50 % of the dipsogenic activity of the octapeptide when infused into the angiotensin sensitive regions in the rat s diencephalon In the present study des_1-angiotensin II appeared dipsogenic although to a lesser degree However the data cannot be readily compared In the goat intraventricular infusion of des_1-$ileu^5$-angiotensin II was used whereas Fitzsimons (1971) applied des_1-val^5-angiotensin II directly into the brain tissue

In the present study the same latency time was observed for the antidiuresis induced by angiotensin II and III This was taken as indication that both polypeptides act on a common presumably membrane located receptor Gagnon Sirois and Park (1975) have studied <u>in vitro</u> the neurohypophyseal receptors for angiotensin II and suggested that cyclic AMP may be involved in the stimulation of ADH secretion from incubated rat neurohypophyses They also found that a much longer incubation period was needed with des_1-angiotensin II than with angiotensin II before ADH was released They assumed that the altered binding affinity for the receptor may contribute to a delay in the action Following longer incubation periods the heptapeptide was capable of producing neurohypophyseal effects comparable to those induced by angiotensin II After intravenous injection of labelled angiotensin II high concentrations of radioactivity were found in the pituitary (Osborne et al 1971 Johnson 1975) This may be one route of entry for the blood-borne angiotensin into the CNS particularly as a releaser of ADH These studies raise the question of the possible existence of two kinds of angiotensin receptors affecting ADH rele se There probably are receptors higher up in the hypothalamic neurohypophyseal system; In addition the neurohypophysis itself may contain receptors

Several reviews have recently been published concernig the central actions of angiotensin II (Severs and Daniels-Severs 1973) central control of fluid homeostasis (Andersson 1974) and control of drinking (Fitzsimons 1972 Peters Fitzsimons and Peters-Haefeli 1975) Therefore only aspects closely related to the present study will be discussed in this chapter

The current opinion is that the fluid balance is principally controlled by osmometric and volumetric mechanisms acting synergistically to maintain the composition and volume of the body fluids There is much evidence that atrial and some other vascular receptors monitor the blood volume and exert a reflex inhibitory tonus on the release of ADH (for review see Gauer Henry and Behn 1970 Goetz Bond and Bloxham 1975) The renal renin-angiotensin system forms an important humoral link in the volume regulation by its influence on aldosterone secretion but apparently also by its effect on central mechanisms involved in the control of fluid balance Experiments in the goat indicate that these centrally mediated effects of angiotensin II are Na^+ dependent Therefore it has been suggested that angiotensin II may act by sensitizing periventricular Na^+ receptors to existing CSF $[Na^+]$ (Andersson 1971) Angiotensin II appears to be dipsogenic in all species tested However many consider that the doses of angiotensin which have been studied are unphysiologically high (e g Abraham et al 1975) Thus the question to what degree the renin-angiotensin of renal origin contributes to the volumetric control of thirst remains open

Various species of animals have been used in the studies concerning the central control of fluid balance This may have been a factor causing some of the divergencies in the results obtained The goat like other ruminants has the voluminous forestomachs acting as a large reservoir for water and electrolytes The dog in contrast is a monogastric carnivore which eats at long intervals Furthermore the modulating effect of anesthetics and surgical stress should be kept in mind

Osmoreceptors versus sodium sensitive receptors

The main evidence for the osmoreceptor theory in the central control of water balance has been obtained by studying the effects of altered solute composition of the blood plasma (Verney 1947) Later studies have given additional support for this concept (see Blass 1973) In Verney s original investigations the blood-brain and the blood-CSF barriers have been disregarded However the responses to the administration of various hypertonic solutions into the central nervous system of the goat from either side of the blood-brain barrier seem to contradict the osmoreceptor theory A rise in the carotid blood osmolality obtained by infusion of NaCl or fructose acts as a much more potent stimulus for ADH release and thirst than the equivalent rise elicited by infusions of urea glycerol or glucose (Eriksson Fernández and Olsson 1971 Olsson 1972) although a rather effective blood-brain barrier seems to exist for all of the latter substances (Crone 1965 Yudilevich and de Rose 1971) On the other hand the infusion of hypertonic sucrose solution into the third cerebral ventricle elicits neither ADH release nor thirst in contrast to corresponding infusion of hypertonic NaCl (Olsson 1969) These observations make the existence of osmoreceptors unlikely at least inside the blood brain barrier The other possibility that cerebral osmoreceptors are outside the blood brain barrier or in a region where the barrier is lacking also appears unlikely Slow infusions of iso- or hypertonic solutions of non-electrolytes into the lateral ventricle repress the dipsogenic antidiuretic and natriuretic responses to intracarotid infusions of hypertonic NaCl (Olsson 1973) This would hardly be the case if th intracarotid infusion of hypertonic NaCl acted <u>via</u> receptors located outside the blood brain barrier

A possible alternative to hypothalamic osmoreceptors may be a sodium sensitive receptor system in the close vicinity of the third cerebral ventricle (<u>cf</u> And rsson 1971) The following results have been taken a evidence for this concept Slow infusions of hypertonic NaCl solution into the third or lateral ventricle elicit drinking antidiuresi and natriuresis in hydrated goats (<u>cf</u> Andersson and Olsson 1973) However no

drinking or antidiuretic responses are obtained by similar in*fusions of hypertonic sucrose (Olsson 1969)* Rather the lowering of CSF $[Na^+]$ by infusing saccharide solutions caused a water diuresis in the non-hydrated goat (paper II) This also happened on most occasions when saccharides were infused together with K^+ Ca^{2+} and Mg^{2+} (paper III) whereas during infusions of isotonic NaCl or saccharides in isotonic NaCl water diuresis did not develope (paper II) The results of combined intracarotid/intraventricular infusions also support the Na^+-receptor hypo*thesis Infusions of saccharides into the lateral ventricle sup*pressed the dipsogenic antidiuretic and natriuretic *responses* to intracarotid infusions of hypertonic NaCl Corresponding infusions of 0 14 M NaCl or isotonic glucose in 0 14 M NaCl did not have this suppressive effect (Olsson 1973)

McKinley Blaine and Denton (1974) reexamined the concept of brain osmoreceptors in thirst regulation in the conscious sheep by making injections into the third ventricle of hypertonic NaCl and saccharide solutions in artificial CSF in an attempt to minimize apart from osmolality the alteration in CSF composition Hypertonic saccharide solutions in water did not elicit drinking while hypertonic saccharides in artificial CSF hypertonic NaCl in artificial CSF and hypertonic NaCl in water were dipsogenic These results were taken as support for combined cerebral osmotic and sodium sensitivity Thus the studies in the sheep lend support to the osmoreceptor theory but do not rule out specific sodium sensitivity since hypertonic NaCl in artificial CSF caused much more drinking than hypertonic saccharides in artificial CSF

The entry of angiotensin II into the brain and the angiotensin sensitive areas

It appears that in order to act centrally circulating angiotensin II must enter the brain However the blood brain barrier effectively restrains angiotensin II (Volicer and Loew 1971 Osborne et al 1971) The other possible routes are via the choroid plexuses into the CSF or an action in areas where the blood-brain barrier is lacking There is no consensus of opinion as to whether angiotensin II penetrates the *blood-CSF* barrier

Volicer and Loew (1971) studied the distribution of ^{14}C-angiotensin II in the brain of mice after systemic intravenous injection The volume of distribution was larger than the plasma volume Furthermore autoradiographs showed radioactivity in choroid plexuses in the lateral and third ventricles and in the aqueduct but not in the fourth ventricle The results were however criticized (Abraham 1974) because of the supraphysiological doses of angiotensin and the incubation method used Using lower doses of tritiated angiotensin II of relatively high specific activity Johnson (1975) studied in detail the distribution of intravenous angiotensin in rat brain Radioactivity was high in the ventricular spaces periventricular tissues choroid plexuses pituitary pineal subfornical organ (SFO) and area postrema Abraham (1974) found the CSF of normal sheep to contain angiotensin II but the levels were not elevated above 5 ng/100 ml when the blood angiotensin II con centrations were raised up to 15-70 ng/100 ml by intravenous infusion As a possible explanation a rapid uptake of angiotensin II from the CSF in physiological situations was taken into consideration

Among the areas where a blood-brain barrier is lacking the area postrema SFO and pituitary are of special interest There is much evidence that blood borne angiotensin II stimu lates central pressor activity in the area postrema whereas the periaqueductal grey (subnucleus medialis) is the site where angiotensin applied into CSF raises blood pressure (for review see Severs and Daniels-Severs 1973)

Circulating angiotensin II may act directly on the neurohypophysis since high radioactivity was observed in the pituitary after intravenous injection of labelled angiotensin II (Osborne et al 1971 Johnson 1975) The finding of high renin (Haulica et al 1975) and angiotensin converting enzyme activity (Yang and Neff 1972) in the hypophysis points to the possibility that also local production of angiotensin II may play a role in the release of ADH Gagnon et al (1975) have studied the rat neurohypophysis <u>in vitro</u> and observed that angiotensin II stimulates ADH secretion possibly <u>via</u> cyclic AMP

The SFO has been claimed by Simpson and Routtenberg (1973) to be the most sensitive locus to angiotensin as a dipsogen

This periventricular organ is in close contact with both blood and CSF Although there is much evidence supporting this concept the SFO apparently is not the only site where angiotensin elicits thirst It is for instance possible to stimulate drinking in spite of massive damage of the SFO if the entry of angiotensin into the anterior part of third ventricle is guaranteed (Buggy et al 1975) During remapping of angiotensin sensitive brain loci Johnson and Epstein (1975) observed that angiotensin II induced thirst if cannula traversed the anterior cerebral ventricles thus allowing the injected material to enter the ventricular system On the basis of this result and of autoradiographic findings (Johnson 1975) they maintain that angiotensin II may use the ventricular route to gain access to the sensitive periventricular sites

It has to be emphasized that in the earlier experiments high doses of angiotensin II were used However Epstein and Hsiao (1975) and Trippodo McCaa and Guyton (1976) claimed on the basis of their recent studies that angiotensin II acts dipsogenically also in physiological doses In addition to the blood-borne angiotensin the existence of the intrinsic renin-angiotensin system in the brain (Ganten et al 1971 Fischer-Ferraro et al 1971) has focused interest on the possible physiological role of central effects of angiotensin The hypothalamic region close to the third ventricle and the brain stem contain the highest level of angiotensin I and II (Fischer-Ferraro et al 1971) The centers which control fluid balance and blood pressure are located here Different regimens of treating animals were found to change the renin activity and angiotensin I content of the brain Systemic administration of aldosterone decreased cerebral renin activity (Ganten et al 1971) On the other hand, administration of hypertonic NaCl (Maulice et al 1975) or $sar^{1}ileu^{8}$-angiotensin II an angiotensin II antagonist (Sen Ferrario and Bumpus 1974) increased this activity Furthermore hypovolemia increased the concentration of angiotensin I in the brain stem whereas Na^{+}-loading decreased it (Slaven 1975) Taken together these data would indicate that systemic changes in the electrolyte and fluid balance affect not only the renal but also the cerebral renin release

Cerebral receptors controlling renal Na^+ excretion

Most studies concerning the central control of Na^+ homeostasis favor the view that a Na^+-sensitive system in the brain operates in the regulation of the renal Na^+ output Elevation of the Na^+ concentration in the CSF of the cerebral ventricular system has been found to increase renal Na^+ excretion in many species Perfusion experiments with low $[Na^+]$ CSF indicate that reduced basal activity of these receptors may play a role in initiating Na^+-sparing during salt depletion The physiological basis for this concept lies in a study with Na^+-depleted and normal sheep (Mouw et al 1974) Progressive Na^+-depletion was associated with a significant reduction of CSF $[Na^+]$ and the Na^+ concentration in CSF and blood were closely related In pentobarbital anesthetized dogs (Mouw and Vander 1970) and in conscious sheep (Mouw et al 1974) hypotonic low $[Na^+]$ perfusion of the cerebroventricular system caused antinatriuresis and a rise in plasma renin In the present work in conscious goats CSF $[Na^+]$ was lowered by ICV infusions of isotonic fructose The PRA rose in accordance with the previous studies but renal Na^+ excretion showed no consistent changes Instead urine flow rate increased and water diuresis developed Similar responses in urine excretion have been reported by Leusen and Lacroix (1961) in chloralose-anesthetized dog during low $[Na^+]$ perfusion of ventricular system In some of their sheep Mouw et al (1974) also observed a marked increase in urine flow during low $[Na^+]$ perfusion and in these instances Na^+ excretion did not decrease In some way the behavior of renal Na^+ excretion thus appears to depend on the degree of diuresis One possibility is that the presence of ADH may be an important factor in renal Na^+-sparing In the present work renal K^+ excretion was found to decrease to some extent This may reflect a diminished Na^+ load at the distal nephron

Intracarotid infusions have also been used to study the postulated central Na^+ receptors Such infusions of hypertonic NaCl solution always indu e a more pronounced natriuresis than equivalent intravenous infusions In the conscious goat intra carotid infusion (1 5 ml/min) of hypertonic (2 M) fructose had a negligible natriuretic effect when compared with hypertonic

(1 M) NaCl infused at a same speed (Olsson and Kolmodin 1974) Similarly in anesthetized cats (Thornborough Passo and Rothballer 1973) intracarotid infusion of hypertonic sucrose in normal saline and in anesthetized dogs (Zucker and Kaley 1976) infusion of hypertonic glucose failed to increase renal Na^+ excretion In conscious sheep on the other hand Blaine et al (1975) observed a definite natriuresis following intracarotid infusion (1 6 ml/min) of hypertonic sucrose or fructose (2 M) but the response to intravenous infusion was delayed and weak Hence the latter authors expressed the belief that osmoreceptors control the Na^+ excretion

The efferent pathway from the brain to the kidney mediating the effects on Na^+ excretion has not been ascertained There are several possible partly interrelated mechanisms These are

(1) alterations in renal hemodynamics including vasomotor autoregulation
(2) changes in renal nerve activity and
(3) humoral factors such as aldosterone ADH and natriuretic factor

1 The natriuresis following elevated CSF $[Na^+]$ in the third ventricle is accompanied by a rise in the glomerular filtration rate (Andersson et al 1969 Zucker Levine and Kaley 1974) In the present study the infusion of hypertonic NaCl into the third ventricle caused a slowly developing rise in blood pressure concurrently with the natriuretic response (paper I) Similar results have been reported in anesthetized rats (Dorn et al 1969) cats (Chiu and Sawyer 1974) and dogs (Zucker et al 1974) The changes in renal hemodynamics may thus contribute to the increased Na^+ excretion (cf Guyton et al 1974) On the other hand the rise in blood pressure following intracarotid infusion of hypertonic NaCl is inconsistent and not well correlated to natriuresis (Blaine et al 1975 Thornborough et al 1973 Zucker and Kaley 1976) Furthermore during antinatriuresis following the lowering of CSF $[Na^+]$ there were no significant changes in blood pressure The redistribution of renal blood flow is another possible mechanism often discussed but not yet established

2 There is much evidence that the renal nerves may be involved in the tubular reabsorption of Na^+ (e g Slick et al 1975; for review see Gill 1969) However the evidence that the changes in Na^+ excretion following stimulation of brain Na^+ receptors are mediated via renal nerves is meager By means of renal denervation McKinley Blaine and Denton (1973) were able to inhibit the natriuresis following the injection of hypertonic NaCl into the third ventricle of conscious sheep However in anesthetized cats (Chiu and Sawyer 1974) and dogs (Zucker et al 1974) renal denervation did not prevent an increase in Na^+ excretion after infusion of hypertonic NaCl into the third ventricle Furthermore the antinatriuresis during low $[Na^+]$ CSF perfusion was not abolished by renal denervation (Mouw and Vander 1971)

3 Although the plasma levels of aldosterone decrease during elevated CSF $[Na^+]$ (Abraham et al 1976) this apparently is not essential for natriuresis since natriuresis develops rapidly and aldosterone pretreatment does not inhibit the response (Andersson et al 1969) During low $[Na^+]$ CSF perfusion blood aldosterone concentration was unchanged (Mouw et al 1974) Thus aldosterone probably did not mediate the antinatriuretic response

Stimuli which elicit natriuresis are known to release ADH While high doses of ADH are natriuretic the importance of this hormone for Na^+ excretion has been much debated (e g Macfarlane et al 1967 Blaine et al 1975) The release of ADH however cannot alone explain the effect since experimental diabetes insipidus does not abolish the natriuretic response (Andersson et al 1969) Pretreatment with ADH like that with aldosterone could not inhibit the natriuretic effect following intracarotid infusion of hypertonic NaCl (Thornborough et al 1973 Zucker and Kaley 1976) Lowering of CSF $[Na^+]$ was in most cases found to decrease ADH release and this may have contributed to the antinatriuresis observed (Mouw et al 1974) However in anesthetized dogs antinatriure i developed even though the plasma ADH concentration was high (Mouw and Vander 1971)

In spite of an intense search for a natriuretic factor conclusive p oof of its existence is still lacking (Nashat 1974)

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APPARENT REGIONAL [illegible] ACETYLCHOLINE IN [illegible]

METHODOLOGICAL AND FUNCT[illegible]

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1 Introduction

The term turnover is defined as the renewal of a substance at steady state (Zilversmit *et al.* 1943).

Measurements of the turnover of a neurotransmitter such as acetylcholine (ACh) in brain regions will give more information about functional relationships than will be obtained by measuring static levels. A change in the release, for example, may leave the steady state unaltered but affect the rate of biosynthesis. Turnover changes in different brain regions may be masked in whole brain studies.

To measure the ACh turnover choline (Ch) may be given intravenously and the time course of the specific radioactivities (SA) of the precursor (Ch) and product (ACh) followed in the brain. Labelled choline (Schuberth *et al.* 1969 Jenden 1973, Saelens *et al.* 1974) or phosphorylcholine (PhCh) (Cheney *et al.* 1975*b*) has been given to mice and rats either by intravenous (i.v.) pulse injection (Schuberth *et al.* 1969 Haubrich *et al.* 1975) or intravenous infusion (Saelens *et al.* 1973*b* Racagni *et al.* 1974).

This method necessitates the assumption that the brain handles the Ch in a way which may be described in terms of an open single compartment model, that there is instant mixing of the labelled and unlabelled compounds and that steady state conditions prevail (Zilversmit 1960).

The turnover values obtained will necessarily be rather approximate. The most obvious limitation is our lack of knowledge about the compartmentation of Ch and the size of the Ch pool available for ACh synthesis (Sparf 1973 Jenden 1976).

In order to avoid this uncertainty Domino and Wilson (1972) measured the ACh turnover from the rate of depletion of the ACh after intracerebroventricular administration of hemicholinium (HC 3), a quaternary drug which inhibits ACh synthesis by blocking Ch transport. Richter and Crossland (1949) measured the initial rate of restitution of the ACh after depletion by electroshock. However in these cases steady state conditions were *not* obtained.

Both the ACh and Ch levels in brain are extremely labile (for review see MacIntosh and Collier 1976). This is a serious obstacle and a limiting factor for work on brain regions. During recent years great progress has been made in the development of methods of getting rapid inactivation of brain enzymes without preventing accurate dissection. When the present study was initiated in 1971 Stavinoha and co-workers (1970) had recently introduced microwave irradiation as a rapid method of enzyme inactivation and sacrifice of small rodents, a method which allowed accurate dissection of the brain.

The aims of the present investigation were:

1 to evaluate the importance of the time of enzyme inactivation for ACh and Ch values in brain tissue;
2. to study the precursor-product relationship of Ch and ACh in different brain regions

by following the time courses of the specific radioactivities of Ch and ACh after an intravenous tracer dose of Ch

3 to apply the data to different models of turnover calculation

4 to study the functional role of ACh in different brain regions by measuring the effect of barbiturates and oxotremorine on the apparent regional ACh turnover

2. Microwave Irradiation as a Rapid Method of Enzyme Inactivation and Sacrifice of Small Rodents

2.1 *Some basic facts*

For detailed information about microwave physics, see Püschner (1966), Thomas (1972), Copson (1962) and Okress (1968).

Microwaves are electromagnetic waves with a wavelength varying between 0.001 and 1 m. The most commonly used wavelengths have dimensions of 0.328 and 0.122 m (915 and 2450 MHz).

Non-metallic materials can be heated by microwave irradiation, since the energy-rich electromagnetic waves can pass into materials and convert their energy into heat. For microwave heating a system consisting of at least three components is necessary: 1) a generator of microwave energy, 2) a waveguide and 3) an applicator.

2.1.1 *Generation of microwaves*

For the generation of microwaves with an effect of less than 10 kW a magnetron is used. This consists of a vacuum diode with a cathode in the centre surrounded by a cylindrical anode containing several resonant cavities (Fig. 1). The cathode consists of a material which emits electrons at increased temperature. The emitted electrons are affected by the force from both a magnetic field (parallel to the cathode) and an electric field (applied voltage between anode and cathode). The electrons are only attracted to the parts of the anode which are positively charged by the electromagnetic oscillation. Subsequently in the interaction space between cathode and anode the emitted electrons travel in spiral paths to form the spokes of a wheel which rotate. In the resonance cavities high frequency oscillation microwaves are excited by the passing spokes of electrons. The generated microwaves can then be transmitted from one of these resonance cavities to the waveguide.

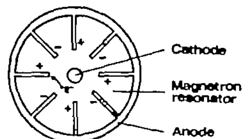


Fig. 1 Magnetron, a diagrammatic drawing.

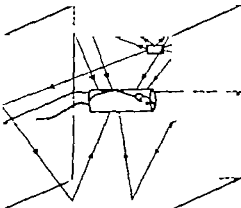


Fig. 2a. Applicator for whole body irradiation of mice. The electromagnetic waves are reflected by the walls of the exposure chamber (oven) to penetrate into the mouse body

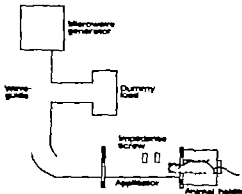


Fig. 2b. Diagrammatic drawing of the microwave system Metabostat 4104 T. The mouse's head is placed in the applicator and exposed to microwaves.

2.1.2 *Waveguide*

The waveguide can be a rectangular metal channel, with the same dimension as the wavelength of the microwaves and constitutes the transmission line for propagation of the microwaves from the generator to the applicator

2.1.3 *Applicator* = exposure chamber (Figs. *2a* *2b*).

2.2 *Heating by microwaves*

A dielectric material with polar properties may be regarded as an electric dipole with a positive and a negative charge (q) separated by a distance l (Fig. 3). If an electric field is applied, the dipole will be oriented in the direction of the field and a dipole moment will be induced ($q \times l$). A change in the direction of the electric field will alter the orientation of the dipoles. If the electric field alternates with a high frequency the dipoles will also oscillate very rapidly and by friction will convert oscillation energy into heat. The dipoles respond with a short delay to the change in the electric field and during this relaxation time the dielectric loss occurs. There are also free charged particles which are able to produce heat by friction. The heat produced per volume can be described by the following equation, where the first term represents the dielectric heat and the second the joule heat.

$$p = \frac{E^2 \cdot \pi \cdot f \cdot \epsilon \cdot \epsilon_0 \tan\delta}{2} + E^2 \cdot \sigma \qquad (1)$$

dielectric heat joule heat

p = power
E = electric field
f = frequency
ϵ = relative permittivity
ϵ_0 = permittivity of vacuum
$\tan\delta$ = loss factor
σ conductivity

A −

+q

E ℓ

+ −q

Fig. 3. Orientation of a dipole in an electric field (E). The positive and negative charges of the dipole (q) are separated by a distance *l*.

The ϵ value is a characteristic of the material and is determined by the force and number of dipoles per volume. Permanent dipoles, *e.g.* water are independent of an electric field. The value for the loss factor ($\tan\delta$) is determined by the friction of the dipoles.

By the conventional heating methods the surface of the material first becomes heated, and the deeper parts are then heated by means of conduction and convection. With microwave irradiation, electromagnetic energy passes into the material more rapidly and more uniformly than the passage of heat by conduction and convection. However the amount of microwave energy delivered decreases during penetration. From the following equation the depth at which the developed heat has decreased to 14% can be calculated.

$$\frac{c}{\pi \; f \; \sqrt{\epsilon} \; \tan\delta} = \text{depth of penetration} \qquad (2)$$

c = velocity of light
f = frequency
ϵ = relative permittivity
$\tan\delta$ = loss factor

It is seen that the depth of penetration is inversely related to the frequency. The dielectric data change with frequency, temperature, and salt concentration. The depth of penetration into water at 2450 MHz is about 11 mm and in a solution of 0.1 M NaCl about 9 mm.

2.3 *Sacrifice of small rodents by microwave irradiation*

In study III a Husqvarna 241● oven with an effect of 1.3 kW and a frequency of 24[illegible] MHz was used for sacrificing mice weighing about 20 g, by whole bod[illegible]. The mouse was held in a plastic holder and placed in the middle of the ov[illegible] always in the same position and with the same orientation. The [illegible] are reflected by the walls and thus penetrate from many direc[illegible] A mode stirrer "stirs" the waves and distributes them more [illegible] simplified illustration of the process is given in Fig. 2a. A fa[illegible] tained between the duration of exposure and the brain [illegible]

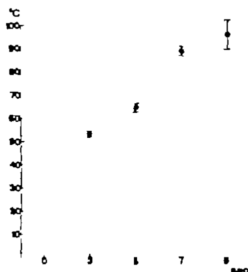


Fig. 4 Mice were sacrificed by whole body microwave irradiation (1.3 kW) for 3, 5 7 or 9 s or by dislocation of the spine. The brain temperature was measured within 11 s. Each point represents the mean of 3–5 experiments. Vertical bars indicate S.E.

completion of the exposure (Fig. 4). The mice are dead after 3 s of exposure (brain temperature 53°C), while denaturation of proteins is found after 7 s of exposure (brain temperature 89°C). The body weight is crucial. The energy required to heat a mouse weighing 20 g from 38°C to 89°C within 7 s can be calculated from the following equation (Lin *et al* 1973)

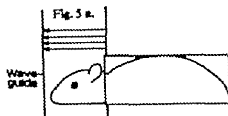
$$W = \frac{\Delta T \cdot 4186 \cdot c \cdot \rho}{t} \qquad (3)$$

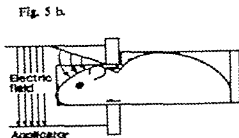
ΔT = change in temperature (°C)
W = absorbed power density (mW g^{-1})
c = specific heat (J g^{-1} °C^{-1})
ρ = density (g cm^{-3})
t = time of exposure (s)

With c = 0.83 J·g °C and ρ = 0.96 g cm as given by Lin *et al* (1973) (using a phantom modelling material for brain tissue) it is found that 0.5 kW is required (38% of the 1.3 kW generated by the magnetron).

To be able to increase the brain temperature even more rapidly Stavinoha *et al* (1973) modified a conventional microwave oven and placed the animal so that the head lay in the centre of the waveguide and the microwaves were admitted on the top of the head (Fig. 5*a*). Essentially the same arrangement has since been used by Guidotti *et al.* (1974), Haubrich and Wang (1976) and Schmidt (1976).

A microwave system (Metabostat 4104 Gerling Moore, California, USA maximal power 5 kW 2450 MHz) specially built for sacrificing small rodents was used in studies III IV and VII (Figs. 2*b*, 5*b*). The animal holder consists of a small tube to hold the head and a larger one for the rest of the body Only the small tube with the head can be inserted into the applicator (Fig. 5*b*). Owing to the design of the applicator the brain will be exposed preferentially to the microwaves. Microwaves that are not absorbed by the head





are reflected to a dummy load (containing water) in order to prevent damage to the magnetron by returning microwaves. To ensure that maximal power will be absorbed by the head, the impedance match between the power source and the head must be optimized. This adjustment (tuning) can be performed by exposing an animal to low-efficiency microwaves (10 mW) and adjusting the impedance screws on the applicator until a minimum of power is reflected toward the power source.

According to equation 3, the depth of penetration (f=2450 MHz) into the head will be about 1 cm.

As described in paper III, a brain temperature of 85–90°C is obtained in the frontal cortex 10 s after termination of a 0.25 s exposure of 5 kW. A comparable brain temperature was obtained in the rat by Butcher *et al.* (1976) with the same type of instrument after 1.7 s of exposure. Theoretically (eq. 3), 3.7 kW is required to increase the temperature of the head of a mouse (2.5 g) by about 50°C in 0.25 s.

A somewhat different type of high power microwave instrument has recently been constructed by Stavinoha and co-workers (Modak *et al.* 1976). By exposure of the head of a mouse for 0.30 s a brain temperature of 90°C is obtained. With their instrument the microwaves are admitted on the top of the head. The instrument allows an individual adjustment of the impedance match for each mouse (Stavinoha 1976).

2.4 *Brain temperatures following microwave irradiation*

The relation between exposure time and brain temperature is shown in Fig. 4 and in paper III. It was not possible to measure the temperature at a shorter interval than 10 s after completion of the irradiation, as the animal had to be taken out of the animal holder, the vault of the skull removed and a thermistor probe inserted into the brain tissue to measure the temperature. The choice of probe is important. Probes with a large diameter tend to give lower temperature readings than those with a smaller diameter. A probe with a diameter of 0.5 mm was used. In study III a brain temperature of 85–90°C was recorded in mice 10–11 s after completion of both whole body irradiation for 7 s (1.3 kW) and irradiation of the head for 0.25 s (5 kW). The temperature then decreased by about 5°C per 15 s during the interval 10 to 45 s after irradiation. It may therefore be assumed that the brain temperature might have been somewhat higher immediately after completion of irradiation. There was no difference in the time course of the brain temperature between the two methods of microwave exposure (paper III).

With the aim of discovering possible temperature gradients due to uneven heating of

the brain, the time course of the temperature was followed in different brain regions (paper III). The temperature was measured at four well-defined points at three different depths at intervals of 15 to 60 s after termination of exposure. Readings from different points at the same depth tended to show the same temperature. However readings from the superficial structures (depth 1 mm) were lower (about 10 per cent) than those from deeper structures (depth 1.5 mm, 2.5 mm). There are two possible explanations Central parts, such as the striatum, may be more efficiently heated than outer parts, such as the cortex and medulla oblongata. This could happen after irradiation of the head with the microwaves "focused" on the head. However these lower outer temperatures were found after both types of exposure (paper III, Nordberg 1976, unpublished observations). The second explanation therefore seems more plausible, namely that the superficial parts cool more rapidly However this is to some extent speculative, as temperature gradients may have occurred before the first time point of measurement. These possible gradients might have been levelled out by conduction of heat during the initial 10—15 s.

In studies III, IV and VII the brain temperature was measured routinely in all animals at a well-defined site. Only brain tissues with a temperature of 85—90°C were included in further analyses (about 2/3 of the irradiated animals).

2.5 *Enzyme inactivation following microwave irradiation*

Most enzymes are thermolabile and irreversibly inactivated at high temperatures. Studies have shown that acetylcholinesterase (AChE) is completely inactivated at 70°C (Silver 1974) and incubation of brain homogenates at 90°C for some seconds inactivates both AChE and choline acetyltransferase (CAT) (Stavinoha *et al.* 1973). Following microwave irradiation of the mouse or rat, almost complete inactivation of brain CAT and AChE has been noted when the brain temperature has increased to 70—90°C (Schmidt *et al* 1972, Stavinoha *et al* 1973 Butcher *et al.* 1976, Schmidt 1976 paper III).

It is a disadvantage that the enzyme activities cannot be measured at the very instant of completion of microwave irradiation. In study III the enzyme activities were measured 1—2 min after termination of irradiation. With use of the low power instrument (paper III) it cannot be excluded that the enzymes might be active during a part of the 7 s exposure time. The enzyme activities might even be accelerated during some part of the temperature increase. This is very difficult to measure. In addition it is possible that the enzymes may be more stable *in vivo* than can be observed in studies *in vitro* (for CAT see Prempeh *et al* 1972). From this point of view it would seem advantageous to shorten the irradiation time from 7 s to 0.25 s.

The influence of the time of enzyme inactivation on values of ACh and Ch in brain tissue was investigated in study III by using three methods of sacrifice, viz. *dislocation of the spine* (7 min before inactivation of enzymes in trichloroacetic acid (TCA)), *whole body microwave irradiation for 7 s* (1.3 kW brain temperatures 85—90°C 11 s after completion of irradiation), and *irradiation of the head for 0.25 s* (5 kW; 85—90°C 10 s

after completion of irradiation). Although it is not known whether the time of enzyme inactivation is absolutely identical with the duration of microwave irradiation, the three inactivation times will be referred to in the following as 7 min, 7 s and 0.25 s.

3. Steady State Levels of Acetylcholine in Brain Tissue

Decapitation with rapid dissection, and freezing in liquid nitrogen (N_2), have until recently been the methods of sacrifice for measurement of the steady state concentrations of ACh in the brains of small rodents. From the time of decapitation until the brain tissue is homogenized and extracted in an acid medium, the enzymes acetylcholinesterase (AChE) and choline acetyltransferase (CAT) are active and may cause changes in the ACh level *post mortem*. The time that elapses before the enzymes are inactivated is therefore probably critical. The time taken for decapitation, dissection and homogenization of whole mouse or rat brain cannot be reduced to less than 30–60 s. The interval before enzyme inactivation can be shortened somewhat by freezing. The brain is frozen *in situ* by dropping the whole animal into liquid nitrogen (−196°C), or the animal is decapitated and the head frozen. The time required for freezing depends on the size. The superficial parts of the brain are cooled more rapidly than the deeper parts. Takahashi and Aprison (1964) immersed rats weighing about 200 g into liquid nitrogen and found that the temperature of the cerebral cortex had dropped to 0°C after 24–40 s, while in the thalamus the corresponding time was 68–83 s. On submersion of the head and thorax a temperature of 0°C was reached in the thalamus after only 31 s (Stavinoha *et al* 1967). Mice are cooled more rapidly and the cerebral cortex, thalamus, and pons medulla reach a temperature of 0°C after 2–4 s, 6–13 s and 13–24 s, respectively (Ferrendelli *et al.* 1972).

Recently brain blowing has been developed (Veech and Hawkins 1974). By an air blast (1–1.5 atm. pressure), the brain tissue between the olfactory bulbs and the superior colliculi is removed and frozen in liquid nitrogen. The whole procedure takes less than 1 s.

To be able to dissect the brain into different regions, Takahashi and Aprison (1964) introduced the near freezing technique. By dipping the rat into liquid nitrogen for only 10 s, the brain is cooled to almost 0°C without being frozen.

Table I summarizes data from the literature on ACh in *whole brain* following four different methods of sacrifice, namely decapitation, freezing, near freezing, and brain blowing. It is seen that values ranging from 7 to 31 nmol g^{-1} brain tissue have been reported and results obtained by different research groups have varied even when the same method of sacrifice has apparently been used. However different extraction media have been used and this is another parameter which might influence ACh values. Toru and Aprison (1966) found higher values in rat brain tissue extracted with formic acid-acetone (31.2 nmol g^{-1}) than when acid-ethanol (21.6 nmol g^{-1}) or trichloracetic acid (15.1 nmol g^{-1}) was used. Domino *et al* (1976) reported higher values with acetonitrile (25.8 nmol g^{-1}) than with acid-ethanol (18.7 nmol g^{-1}).

The method of assay is a third parameter which may affect ACh values. Until ten

years ago, ACh was mainly assayed by biological methods, using such tissues as guinea-pig ileum, the dorsal muscle of the leech and the frog rectus abdominis muscle, for example. Several chemical, gas chromatographic, and radioenzymatic methods have recently been developed. The different assay methods available are listed in Table II, together with the sensitivity limit for each method. As seen in Table I, Stavinoha and Ryan (1965) and Domino *et al.* (1976), when using the same extraction media, obtained higher ACh values in rat brain by gas chromatography (21.0 nmol g^{-1} 18.7 nmol g^{-1}) than by use of guinea-pig ileum (14.6 nmol g^{-1}) and frog rectus abdominis muscle (9.5 nmol g^{-1}).

Differences in species, strain, age and the time of day of sacrifice are further parameters that may influence the ACh values (Saelens *et al.* 1973*a*, Hanin *et al* 1970, Saito *et al.* 1975). Hanin *et al* (1970) reported a higher ACh content in rat whole brain during the light period of the day (39 6 nmol g   2 h of light) than during the dark period (20.5 nmol g   6 h of darkness). This diurnal variation was only observed in decapitated rats and not in rats frozen in liquid nitrogen.

For measuring the ACh content *in discrete brain regions* both decapitation and near freezing have been used. Decapitation followed by dissection of the brain into regions increases the time interval before enzyme inactivation by minutes. More rapid inactivation should be obtained by the near freezing method (Aprison and Takahashi 1964). Table III summarizes some data on ACh in different brain regions obtained by these two methods of sacrifice. No clear difference between the methods is seen and the values vary considerably even with the same method. In the striatum, for example, ACh contents from 23 nmol g^{-1} (rat) to 40 nmol g^{-1} (mouse) have been obtained.

Thus several factors may influence the measured ACh values, and none of them can be disregarded as unimportant (Tables I—III). To improve one of these parameters, the time of enzyme inactivation, the use of the microwave technique as a rapid method of sacrifice has increased progressively during recent years (Stavinoha *et al* 1970, 1973 1974 Schmidt *et al.* 1972, Sethy *et al.* 1973 Butcher and Butcher 1974 1976, Guidotti *et al* 1974 Longoni *et al.* 1974 Haubrich *et al.* 1975 Freeman *et al.* 1975 Racagni *et al.* 1974 Nordberg and Sundwall 1975 Atweh and Kuhar 1976 Modak *et al.* 1976, Sethy 1976, Speth *et al* 1976, Zsilla *et al.* 1976, Cohen and Wurtman 1976, Karlén *et al* 1976, papers III, IV VII).

Table IV presents a summary of published values for ACh in *whole brain* following alternative methods of microwave irradiation. Values for mouse and rat brain vary from 18—29 and 20—25 nmol g^{-1} respectively The values obtained by microwave irradiation are usually somewhat higher than those obtained after decapitation.

There appears to be no clear cut relationship between presumed inactivation time and ACh content in whole brain. The values reported by Stavinoha *et al* (1973) and Modak *et al* (1976) are almost identical (25.5 nmol g   and 24.2 nmol g^{-1}) (mouse) despite the differences in power source and exposure time (1.2 kW for 6 s and 6 kW for 0.30 s).

Stavinoha *et al.* (1973) found an approximately 80 per cent higher content of ACh (25 4 nmol·g^{-1}) in rats following head irradiation (1.2 kW) for 6 s than following decapitation (13 18 nmol g^{-1}). Freeman *et al* (1975) noted a 20 per cent higher ACh

TABLE I *Endogenous ACh in whole brain – comparison of published values (nmol g^{-1})*

Method of sacrifice	Species	Extraction media	Assay method	Content of ACh		Reference
Decapitation	Rat	Acid leech Ringer phosphate eserine	Leech muscle	19.0 ± 2.8	(S.D)	Elliott and Henderson 1951
	Mouse	Perchloric acid	Radioenzymatic	16.3 ± 1.1	(S.E)	Feigenson and Saelens 1969
	Rat		Gas chromatography	28.9 ± 2.95	(S.E)	Hanin et al. 1970
	Rat			20.2 ± 2.9	(S.E)	Sethy et al. 1973
	Mouse		Mass fragmentography	16.1 ± 0.3	(S.E)	Karlén et al. 1975
	Rat		Fluorometric	11.5 ± 2.4	(S.D)	Browning 1972
	Rat	Acid-ethanol	Bioassay Heart of clam	15.2 ± 1.88[a]	(S.D)	Takahashi and Aprison 1964
	Rat		Guinea pig ileum	14.6 ± 0.6	(S.D)	Stavinoha and Ryan 1965
	Rat		Gas chromatography	21.0 ± 0.5	(S.D)	Stavinoha and Ryan 1965
	Rat			18.7 ± 0.4	(S.E)	Domino et al. 1976
	Rat		Frog rectus abdominis	9.5 ± 0.5	(S.E)	Domino et al. 1976
	Mouse	Formic acid-acetone	Radioenzymatic	9.0 ± 0.8	(S.E)	Saelens et al. 1970
	Rat	Trichloroacetic acid	Gas chromatography	13.7 ± 1.70	(S.D)	Stavinoha et al. 1973
	Mouse		Leech muscle	14.4 ± 0.57	(S.E)	Nordberg and Sundwall 1975 (Paper I)
	Rat	Boiling acid Ringer + trichloroacetic acid	Frog rectus abdominis	15.7 ± 0.9[b]	(S.D)	Giarman and Pepeu 1962
	Rat	Acetonitrile	Guinea pig ileum	24.8		Szilagyi et al. 1972
	Rat		Gas chromatography	26.4		Szilagyi et al. 1972
	Rat			25.8 ± 0.4	(S.E)	Domino et al. 1976
Freezing	Rat	Buffered saline	Frog rectus abdominis	6.7 ± 0.7	(S.D)	Richter and Crossland 1949
	Rat		Leech muscle	8.5 ± 1.5	(S.D)	Richter and Crossland 1949

	Rat	Acid saline-eserine	Frog rectus abdominis	17.1 ± 1.9	(S.D)	Crossland et al. 1955
	Rat	Eserinized saline		15.0 ± 1.6	(S.D)	Takahashi et al. 1961
	Rat	Trichloroacetic acid		7.0 ± 1.9	(S.D)	Richter and Crossland 1949
	Rat		Cat blood pressure	10.9 ± 2.4	(S.D)	Richter and Crossland 1949
	Mouse		Leech muscle	17.4 ± 1.0	(S.E)	Lundholm and Sparf 1975
	Rat		Guinea pig ileum	15.1 ± 0.6	(S.D)	Toru and Aprison 1966
	Rat	Acid-ethanol	Heart of clam	18.7 ± 1.25^a	(S.D)	Takahashi and Aprison 1964
	Rat		Frog rectus abdominis	15.8 ± 0.8	(S.E)	Domino et al. 1976
	Rat		Guinea pig ileum	21.6 ± 1.1^a	(S.D)	Toru and Aprison 1966
	Rat	Formic acid-acetone		31.2 ± 1.5^a	(S.D)	Toru and Aprison 1966
	Rat		Radioenzymatic	26.5 ± 2.2	(S.E)	Aprison et al. 1974
	Rat	Perchloric acid	Gas chromatography	15.8 ± 0.77	(S.E)	Hanin et al. 1970
Near freezing	Rat	Acid-ethanol	Heart of clam	18.7 ± 1.58^a	(S.D)	Takahashi and Aprison 1964
	Rat	Formic acid-acetone	Radio-enzymatic	25.7 ± 0.9	(S.E)	Aprison et al. 1974
	Rat			24.2 ± 1.1	(S.E)	Saelens et al. 1973a
	Mouse			14.5 ± 1.0	(S.E)	Saelens et al. 1973a
Brain blowing	Rat	Acetonitrile + trichloroacetic acid	Gas chromatography	19.5 ± 1.2^c	(S.D)	Veech and Hawkins 1974
	Rat	Perchloric acid alt. formic acid-acetone		12 ± 1.8^a	(S.E)	Guidotti et al. 1974

[a] Excluding cerebellum, medulla oblongata, pons

[b] Excluding cerebellum

[c] Brain tissue between the olfactory bulbs and the superior colliculi

TABLE II *Methods for ACh assay*

	Sensitivity pmol	Reference
Frog rectus abdominis	200 – 1000	Marchbank 1975
"	25	Whittaker 1963
Leech muscle	5	Whittaker 1963
Guinea pig ileum	10	Whittaker 1963
Cat blood pressure	5 – 10	Whittaker 1963
Venous heart	0.5	Whittaker 1963
Radioenzymatic	<100	Hanbrich and Reid 1974
"	2	Goldberg and McCaman 1974
	20	Aprison et al. 1974
"	10	Saelens et al. 1973a
Gas chromatography demethylation	50	Jenden and Hanin 1974
Gas chromatography pyrolysis	14 – 138	Green and Szilagyi 1974
Gas chromatography ion pair extraction, demethylation	1000	Karlén et al. 1974
Mass fragmentography ion pair extraction, demethylation	5	Karlén et al. 1974
Polarographic	100	Maslova 1974
Fluorometric	500	Speeg 1974
	100	Browning 1972

content (22.4 nmol g^{-1}) after head irradiation (5 kW) for 1.2 s than after decapitation (18.6 nmol g^{-1}). However following whole body irradiation of rats for 40 s Sethy *et al.* (1973) found no significant change in the ACh content (22.2 nmol g^{-1}) as compared with rats killed by decapitation (20.2 nmol g^{-1}).

ACh levels in mouse *brain regions* were measured with variations in the time of enzyme inactivation (7 min, 7 s and 0.25 s; paper III). A markedly higher value was obtained with an interval of 0.25 s than with a 7 s interval (Table V). All variables, except the method of sacrifice, were kept constant. Following whole body irradiation (7 s, 1.3 kW) a significant increase in ACh was obtained only in the cortex (+ 39%). After the more efficient irradiation (0.25 s), the increase in cortex was markedly higher (+ 83%) and, moreover significantly higher values were obtained in the striatum (+ 103%), medulla oblongata (+ 47%), and midbrain (+ 39%) in comparison with decapitated animals. A markedly higher ACh content has also been found by Weintraub *et al.* (1976) in the striatum and cortex following head irradiation of rats (6 s, 1.2 kW) in comparison with decapitation. In the other brain regions, however lower ACh values were obtained in irradiated rats (Weintraub *et al.* 1976).

Table VI summarizes values for ACh in brain regions obtained by various research groups using different types of microwave equipment and different procedures.

TABLE III. *Steady state concentrations of endogenous ACh in different brain regions – comparison of published values (nmol g^{-1})*

Endogenous ACh nmol g^{-1}	Takahashi and Aprison 1964	Campbell and Jenden 1970	Sethy *et al.* 1973	Nordberg and Sundwall 1975 (Paper I)	Consolo *et al.* 1976
Method of sacrifice and analysis	Near freezing, bioassay heart of clam, venus mercenaria	Decapitation, pentane −5° C, gas chromatography	Decapitation, pentane −5 C, gas chromatography	Dislocation of the spine, bioassay leech muscle	Decapitation + near freezing, radiochemical assay
Brain region	Rat	Rat	Rat	Mouse	Rat
	$\bar{M}$ ± S.D	$\bar{M}$ ± S.E.	$\bar{M}$ ± S.E.	$\bar{M}$ ± S.E.	$\bar{M}$ ± S.E.
Cerebellum	2.3 ± 0.74	4.1 ± 0.5	4.7 ± 0.5	4.3 ± 0.30	3.7 ± 0.5
Brain stem (Medulla oblongata)	12.3 ± 2.39	Midbrain-pons-medulla 22.6 ± 2.6	20.0 ± 0.8	21.6 ± 0.65	
Midbrain	Diencephalon 22.3 ± 1.84	Thalamus 21.7 ± 2.0	27.7 ± 1.3	20.7 ± 1.47	Diencephalon 24.0 ± 0.4
Hypothalamus	Mesencephalon 16.7 ± 2.68	20.5 ± 1.3	34.0 ± 3.8	–	Mesencephalon 19.2 ± 1.0
Striatum	37.3 ± 2.69	23.2 ± 3.8	37.0 ± 3.1	40.1 ± 1.71	28.8 ± 0.7
Hippocampus	–	–	20.4 ± 2.0	17.0 ± 0.90	18.4 ± 0.7
Cortex	–	13.5 ± 1.6	9.8 ± 1.2	13.3 ± 0.95	13.9 ± 0.7

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Leech muscle	5	Whittaker 1963
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Venous heart	0.5	Whittaker 1963
Radioenzymatic	<100	Hanbrich and Reid 1974
	2	Goldberg and McCaman 1974
	20	Aprison et al. 1974
	10	Saelens et al. 1973a
Gas chromatography demethylation	50	Jenden and Hanin 1974
Gas chromatography pyrolysis	14 – 138	Green and Szilagyi 1974
Gas chromatography ion pair extraction, demethylation	1000	Karlén et al. 1974
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Polarographic	100	Mašlova 1974
Fluorometric	500	Speeg 1974
	100	Browning 1972

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Method of sacrifice and analysis	Near freezing, bioassay heart of clam, venus mercenaria	Decapitation, pentane –5° C, gas chromatography	Decapitation, pentane –5° C, gas chromatography	Dislocation of the spine, bioassay leech muscle	Decapitation + near freezing, radiochemical assay
Brain region	Rat	Rat	Rat	Mouse	Rat
	M$\bar{v}$ ± S.D	M$\bar{v}$ ± S.E.	M$\bar{v}$ ± S.E.	M^{-} ± S.E.	M^{-} ± S.E.
Cerebellum	2.3 ± 0.74	4 1 ± 0.5	4.7 ± 0.5	4.3 ± 0.30	3.7 ± 0.5
Brain stem (Medulla oblongata)	12.3 ± 2.39	Midbrain-pons-medulla 22.6 ± 2.6	20.0 ± 0.8	21.6 ± 0 65	
Midbrain	Diencephalon 22.3 ± 1.84	Thalamus 21 7 ± 2.0	27 7 ± 1.3	20.7 ± 1 47	Diencephalon 24 0 ± 0.4
Hypothalamus	Mesencephalon 16.7 ± 2.68	20.5 ± 1.3	34.0 ± 3.8	–	Mesencephalon 19.2 ± 1.0
Striatum	37.5 ± 2.69	23.2 ± 3.8	37.0 ± 3.1	40.1 ± 1 71	28.8 ± 0.7
Hippocampus	–	–	20.4 ± 2.0	17.0 ± 0.90	18.4 ± 0.7
Cortex	–	13.5 ± 1.6	9.8 ± 1.2	13.3 ± 0.95	13 9 ± 0.7

TABLE IV: *Steady state concentrations of endogenous ACh in whole brain following sacrifice by microwave irradiation – comparison of published values (nmol g^{-1})*

Microwave irradiation	Extraction media	Assay method	Acetylcholine (nmol g^{-1}) M ± S.E.		Reference
Whole body irradiation					
Rat (242 g); 1.25 kW; 20 s; 85° C (15–20 s *after completion of* irradiation)	acetonitrile + TCA	gas chromatography	19.91 ± 0.91		Schmidt et al. 1972
Rat (225–275 g); 40 s;	perchloric acid	gas chromatography	22.2 ± 3.2		Sethy et al. 1973
Head irradiation					
Rat (100–200 g); 1.2 kW 6 s; 92° C;	acetonitrile + TCA	gas chromatography	25.4 ± 1.49	(S.D)	Stavinoha et al. 1973
Mouse (18–22 g); 1.2 kW 3 s; 85° C;	acetonitrile + TCA	gas chromatography	25.5 ± 2.63	(S.D)	Stavinoha et al. 1973
Rat (200–280 g); 5 kW 1.2 s;	formic acid-acetone	gas chromatography/ mass spectrometry	22.4 ± 1.4		Freeman et al. 1975
Mouse (20–25 g); 1.3 kW 3–3.6 s;	formic acid-acetone	radioenzymatic	29 ± 3		Hanbrich and Wang 1976
Mouse (20–25 g); 2.0 kW 1.4 s;	perchloric acid	gas chromatography	18 ± 0.56		Trabucchi et al. 1975
Mouse (19–26 g); 2.5 kW 0.62 s;	perchloric acid	mass fragmentography	26.1 ± 4.1	(S.D)	Karlén et al. 1976
Mouse (20–30 g); 6 kW 0.300 s; 90°C;	formic acid-acetone	pyrolysis-gas chromatography	24.2 ± 2.8	(S.D)	Modak et al. 1976

TABLE V: *Endogenous ACh and Ch (nmol g^{-1}) in different mouse brain regions following 3 methods of sacrifice.*

	Acetylcholine			Choline	
	Dislocation of the spine	Microwave irradiation		Microwave irradiation	
		Whole body 7 s	Head 0.25 s	Whole body 7 s	Head 0.25
Cerebellum	3 7 ± 0.38 (11)	4.6 ± 0.97 (3)	5.2 ± 1.05 (4)	39.0 ± 4 49 (6)	18.6 ± 3.37 (3)
Medulla oblongata	19.8 ± 1.07 (12)	20.8 ± 3.01 (3)	29.1 ± 1 10 (4)***	44.0 ± 7.24 (5)	46.7 ± 11.50 (3)
Midbrain	21.1 ± 1.24 (14)	23.2 ± 1 47 (5)	29 4 ± 1.65 (4)**	39.3 ± 4 76 (7)	41.3 ± 7.20 (3)
Striatum	37 1 ± 2 06 (14)	40.4 ± 3.73 (5)[a]	75.3 ± 1.84 (3)[b]***	48.8 ± 6.50 (6)	41 4 ± 5.65 (4)
Hippocampus	15.8 ± 0.83 (14)	17.1 ± 1 77 (5)	20.8 ± 2.25 (4)	43.8 ± 5.37 (6)	27 4 ± 7 63 (3)
Cortex	13.2 ± 0.74 (12)	18.4 ± 1.55 (5)**	24.2 ± 1.52 (4)***	30.4 ± 2.80 (6)	30.5 ± 3.50 (4)

MV ± S.E. (n) number of experiments. * $p<0.05$ ** $p<0.01$, *** $p<0.001$

[a] In an additional sample 70.3 nmol g^{-1}

[b] In an additional sample 48.7 nmol g^{-1}

TABLE VI. *Steady state concentrations of endogenous ACh in different brain regions following*

Endogenous acetylcholine nmol g^{-1}	Schmidt et al., 1972	Nordberg and Sundwall 1976 (Paper III)	Stavinoha et al., 1973 1974
Method of microwave irradiation, tissue extraction and analysis	WHOLE BODY	IRRADIATION	HEAD
	Rat (115 g) 1.25 kW 15 s, 96° (15–20 s after completion of irradiation)	Mouse (18–22 g); 1.3 kW 7 s; 88–89° C (11 s after completion of irradiation)	Rat (200–250 g); 1.2 kW 6 s; 92°C;
	acetonitrile + TCA gas chromatography	TCA leech muscle	formic acid-acetone pyrolysis-gas chromatography
Brain region	$M\bar{v} \pm$ S.E.	$M\bar{v} \pm$ S.E.	$M\bar{v} \pm$ S.D
Cerebellum	6.55 ± 0.93	4.6 ± 0.97	7.2 ± 2.0
Brain stem (Medulla oblongata)	27.37 ± 1.22	20.8 ± 3.01	30.9 ± 4.7
Midbrain	30.69 ± 1.22	23.2 ± 1.47	–
Hypothalamus	34.12 ± 1.67	–	–
Striatum	46.96 ± 2.22	40.4 ± 3.73	55.4 ± 4.7
Hippocampus	24.24 ± 0.51	17.1 ± 1.77	25.8 ± 2.7
Cortex	10.95 ± 0.46	18.4 ± 1.55	21.1 ± 3.0

Although the values for the striatum vary between 40 and 88 nmol g^{-1} it is worthy of note that values from 77 to 88 nmol g^{-1} have been obtained with the presumably more efficient equipment (5 to 6 kW).

…sacrifice by microwave irradiation – comparison of published values (nmol g^{-1})

Modak et al., 1976	Schmidt, 1976	Zaffa et al., 1976	Butcher et al., 1976	Nordberg and Sandwall, 1977 (Paper IV)
IRRADIATION				
Mouse (20–30 g); 6 kW 0.300 s; 90° C;	Rat (150–200 g)· 1.25 kW 2.5 s; 95° C;	Rat (100–120 g)· 2.0 kW 2 s;	Rat (230–250 g) 5 kW· 1.7 s; 87° C;	Mouse (20–24 g), 5 kW 0.25 s; 88° C; (10 s after completion of irradiation)
formic acid-acetone pyrolysis-gas chromatography	toluene-ether pyrolysis-gas chromatography	perchloric acid gas chromatography	formic acid-acetone, gas-chromatography/mass spectrometry	TCA leech muscle
Mv̄ ± S.D.	Mv̄ ± S.E.	Mv̄ ± S.E.	Mv̄ ± S.E.	M̄ ± S.E.
16.6 ± 2.4	6.8 ± 0.2	–	6.5 ± 0.6	5.2 ± 1.05
44.1 ± 1.9	31.9 ± 1.2	34 ± 2.0	31.9 ± 0.9 (including midbrain)	29.1 ± 1.10
33.8 ± 2.2	35.3 ± 2.0	–	–	29.0 ± 1.80
–	32.3 ± 1.3	–	31.3 ± 1.0	–
81.0 ± 1.9	64.4 ± 2.1	51 ± 1.9	88.3 ± 4.0	76.8 ± 1.78
31.0 ± 1.9	29.0 ± 0.6	25 ± 1.6	28.4 ± 0.9	21.2 ± 2.32
25.7 ± 3.4	27.6 ± 1.3	16 ± 1.2	21.8 ± 1.0	24.8 ± 1.44

4. Steady State Levels of Choline in Brain Tissue

The values reported for free Ch in brain vary considerably. Sparf (1973) summarizes the data for free Ch published up to 1973. Values ranging from 600 nmol g^{-1} (Smith and Saelens 1967) to 21.5 nmol g^{-1} (Eade *et al.* 1973) are given. It has been suggested that the rapid *post mortem* increase in free Ch (Schuberth *et al.* 1970*a*) is caused by a breakdown of glycerylphosphorylcholine and/or phosphatidylcholine (Dross and Kewitz 1972, Dross 1975) or to interruption of the venous drainage (Kewitz *et al.* 1973). The lowest values, 21.5–35.0 nmol g^{-1} (Eade *et al.* 1973) and 39.3 nmol g^{-1} (Ewetz *et al.* 1970), were obtained after sacrifice by total freezing and are in fairly good agreement with the value obtained by extrapolation of the *post mortem* increase in Ch (27.5 nmol·g^{-1} Dross and Kewitz 1972) and the *in vivo* concentration of Ch calculated by Hanin and Schuberth (30—40 nmol g^{-1}) from data on dilution of the brain Ch pool by deuterium-labelled Ch (d-Ch).

Near freezing tends to give higher Ch values (64.0 nmol g^{-1} Aprison *et al.* 1974). Following microwave irradiation of the head values of 26.3 nmol g^{-1} (Stavinoha and Weintraub 1974) and 24.2 nmol g^{-1} (Freeman *et al.* 1975) for the rat and 28 nmol g^{-1} (Haubrich and Wang 1976), 33 nmol g^{-1} (Trabucchi *et al.* 1975), 32.4 nmol g^{-1} (Nordberg 1975 unpublished observation) and 49.1 nmol g^{-1} (Karlén *et al.* 1976) for the mouse have been obtained.

Data for Ch in different brain regions following microwave irradiation are given in Table VII. Values between 14 and 45 nmol g^{-1} have been reported. There is no consistent difference between brain regions. The Ch contents of the striatum given in Table VII are much lower than those obtained after decapitation and freezing (152.5 nmol g^{-1} Butcher *et al.* 1976 127 nmol g^{-1} Sethy *et al.* 1973 96.1 nmol g^{-1} Ladinsky *et al.* 1976). In study III no marked difference in the Ch contents of different brain regions was found when the time of microwave exposure was reduced from 7 s to 0.25 s.

TABLE VII. *Steady state concentrations of endogenous Ch in different brain regions following sacrifice by irradiation of the head – comparison of published values (nmol g^{-1})*

Microwave irradiation	Stavinoha et al., 1973 1974	Schmidt, 1976	Butcher et al., 1976	Zalla et al., 1976	Nordberg and Sundwall 1976b (Paper IV)
	Rat; 1.2 kW; 6 s; 92° C	Rat; 1.25 kW; 2.5 s; 95 °C	Rat; 5 kW; 1.7 s; 87° C	Rat, 2.0 kW 2 s;	Mouse; 5 kW 0.25 s; 88° C
Brain region	$\bar{M}$ ± S.D.	$\bar{M}$ ± S.E.	$\bar{M}$ ± S.E	$\bar{M}$ ± S.E.	$\bar{M}$ ± S.E.
Cerebellum	17.6 ± 4.2	30.0 ± 1.8	17.1 ± 2.7	–	32.8 ± 4.63
Brain stem (Medulla oblongata)	34.7 ± 5.8	29.3 ± 1.8	14.3 ± 0.4 (including midbrain)	36 ± 4.4	43.8 ± 4.30
Midbrain	–	28.1 ± 1.6		–	33.8 ± 3.56
Hypothalamus	–	28.2 ± 2.1	26.9 ± 0.4	–	–
Striatum	43.0 ± 1.7	32.0 ± 1.5	24.9 ± 1.7	39 ± 3.4	38.2 ± 3.11
Hippocampus	36.0 ± 9.7	24.0 ± 1.4	26.3 ± 1.5	45 ± 5.5	28.2 ± 3.18
Cortex	22.1 ± 1.8	28.4 ± 2.5	17.6 ± 2.6	26 ± 3.5	33.8 ± 3.34

4. Steady State Levels of Choline in Brain Tissue

The values reported for free Ch in brain vary considerably Sparf (1973) summarizes the data for free Ch published up to 1973 Values ranging from 600 nmol·g^{-1} (Smith and Saelens 1967) to 21.5 nmol g^{-1} (Eade *et al.* 1973) are given. It has been suggested that the rapid *post mortem* increase in free Ch (Schuberth *et al.* 1970*a*) is caused by a breakdown of glycerylphosphorylcholine and/or phosphatidylcholine (Dross and Kewitz 1972, Dross 1975) or to interruption of the venous drainage (Kewitz *et al.* 1973). The lowest values, 21.5—35.0 nmol·g^{-1} (Eade *et al.* 1973) and 39.3 nmol·g^{-1} (Ewetz *et al.* 1970), were obtained after sacrifice by total freezing and are in fairly good agreement with the value obtained by extrapolation of the *post mortem* increase in Ch (27.5 nmol·g^{-1} Dross and Kewitz 1972) and the *in vivo* concentration of Ch calculated by Hanin and Schuberth (30—40 nmol g^{-1}) from data on dilution of the brain Ch pool by deuterium-labelled Ch (d-Ch).

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TABLE VIII. *Turnover rate of ACh (nmol g^{-1} min^{-1}) in whole brain determined by tracer techniques – comparison of published values*

Turnover rate nmol g^{-1} min^{-1}	Species	Labelled precursor	Method of sacrifice	Reference
5 10	Mouse	Intravenous pulse injection of 25 µmol kg^{-1} ^{3}H-Ch	Decapitation	Schuberth et al. 1970b
20	Mouse	Intravenous pulse injection of 25 µmol kg^{-1} ^{3}H-Ch	Liq N_2	Sparf 1973
6.5 16.8	Mouse	Intravenous pulse injection of 35 µmol kg^{-1} ^{14}C-Ch	Liq N_2 3.3 s	Saelens et al. 1974
5 7.5 1 4; 7.9	Mouse	Intravenous pulse injection of 20 µmol kg^{-1} d_4–Ch	Decapitation + liq N_2	Jenden 1973 Jenden 1974 Jenden et al. 1974
6	Mouse	Intravenous pulse injection of 5 1 µmol kg^{-1} ^{14}C-PhCh	Microwave irradiation of the head 1.4 s (2 kW)	Trabucchi et al. 1975

5 Apparent Rate of Turnover of Acetylcholine in Brain Regions

5.1 *Definitions*

Turnover: The renewal of a substance in a tissue at steady state.
Turnover rate: The quantity of a substance in a tissue that is renewed per unit of time.
Turnover time: *The time taken for a whole pool of a substance present in a tissue to be renewed.*

The principles for the use of radioactive precursors for measurements of turnover as formulated by Zilversmit (1960) require the assumption of a system with the following properties:

a. it behaves like an open single compartment
b. radioactive and endogenous substances are handled in the same way by the system
c. no distinction is made by the system between old and newly synthesized substances
d. existence of steady state conditions (the rate of synthesis of the substance equals the rate of disappearance).

5.2 *Previous results*

A summary of published values for turnover of ACh in whole brain or brain regions obtained by the use of tracer techniques is given in Tables VIII and IX. Values obtained by other means are summarized in Table X. With the tracer techniques the turnover rate in whole brain has varied between 1 and 20 nmol g^{-1} min^{-1} (Table VIII). Although different precursors, modes of sacrifice and methods of turnover calculations have been used, each research group has obtained at least one turnover value within the range of 5 to 8 nmol g^{-1} min^{-1}. The value (38 nmol g^{-1} min^{-1}) calculated by Richter and Crossland from the initial rate of resynthesis of ACh after partial depletion of ACh in the brain by electroshock is much higher than all other values given for the whole brain (Tables VIII, X). But it is rather close to the maximal capacity of CAT in 1 g brain tissue to synthesize ACh *in vitro* (60 nmol g^{-1} Schrier and Shuster 1967). For different brain regions (Tables IX, X) turnover rates of between 1 and 55 nmol g^{-1} min^{-1} have been reported. The turnover rate seems to be highest in the striatum (6–55 nmol g^{-1} min^{-1}), although the values differ between research groups (Table IX, X). It is, however, worth noting that the apparent turnover values obtained in paper study IV (Table IX, this survey) by a tracer technique and the values calculated by Stavinoha *et al.* (1976) (Table X) when the accumulation rate of ACh in different brain regions is measured after i.v. injection of dichlorvos (15 mg kg^{-1}) in rats are quite identical.

5.3 *Biotransformation of radioactive choline in brain regions*

Not only the steady state concentrations of ACh and Ch but also the kinetics of tracer doses of ^{3}H Ch might be dependent on the time of enzyme inactivation. A comparison

TABLE VIII. *Turnover rate of ACh (nmol g^{-1} min^{-1}) in whole brain determined by tracer techniques – comparison of published values*

Turnover rate nmol g^{-1} min^{-1}	Species	Labelled precursor	Method of sacrifice	Reference
5 10	Mouse	Intravenous pulse injection of 25 μmol kg^{-1} ^{3}H-Ch	Decapitation	Schuberth et al. 1970b
20	Mouse	Intravenous pulse injection of 25 μmol kg^{-1} ^{3}H-Ch	Liq N_2	Sparf 1973
6.5 16.8	Mouse	Intravenous pulse injection of 35 μmol kg^{-1} ^{14}C-Ch	Liq N_2 3.5 s	Saelens et al. 1974
5 7.5 14.79	Mouse	Intravenous pulse injection of 20 μmol kg^{-1} d_4–Ch	Decapitation + liq N_2	Jenden 1973 Jenden 1974 Jenden et al. 1974
6	Mouse	Intravenous pulse injection of 5.1 μmol kg^{-1} [illegible] C-PhCh	Microwave irradiatio of the head 1.4 s (2 kW)	Trabucchi et al. 1975

TABLE IX. *Turnover rate of ACh (nmol g^{-1} min^{-1}) in different brain regions obtained by tracer techniques – comparison of published values*

Brain region Reference Labelled precursor Method of sacrifice	Cerebellum	Brain stem	Midbrain	Striatum	Hippocampus	Cortex
Saelens et al. 1973b i.v. infusion 0.016 µmol kg^{-1} min^{-1} ^{3}H-Ch, rat, decapitation	–	5.0	8.4	–	–	20.9
Saelens et al. 1974 i.v. injection 35 µmol kg^{-1} ^{14}C-Ch, mouse liq. N_2 3.5	1.34	5.63	6.25	–	–	6.64
Racagni et al. 1974 i.v. infusion 0.63 µmol kg^{-1} min^{-1} ^{14}C-PhCh, 2–8 min, rat, head irradiation, 2 s (2 kW)	–	1.5	–	21.7	–	3.3
Cheney et al. 1975a i.v. infusion 0.63 µmol kg^{-1} min^{-1} ^{14}C PhCh, 6 min, rat, head irradiation, 3 (2.5 kW)	–	–	–	5.7	–	1.4
Zsilla et al. 1976 i.v. infusion 25 µCi kg^{-1} min^{-1} ^{14}C-PhCh, 6 min, rat, head irradiation, 2 s (2 kW)	–	4.2	–	12.7	8.8	3.2
Nordberg and Sundwall 1977 (Paper IV) i.v. injection 0.75 µmol kg^{-1} ^{3}H-Ch, mouse, head irradiation, 0.25 s (5 kW)	(2)	10	11	55	21	27
Haubrich et al. 1975 i.v. injection 0.5 µmol kg^{-1} ^{3}H-Ch, rat, head irradiation, 4.5 (1.3 kW)		–	–	18	–	5

TABLE X. *Turnover rate of ACh (nmol g^{-1} min^{-1}) in brain tissue determined by non-tracer techniques – comparison of published values*

Turnover rate nmol g^{-1} min^{-1}	Brain tissue	Species	Method	Reference
38	Whole brain	Rat	Calculation of the initial rate of ACh resynthesis after partial depletion of the ACh content in brain following electroshock	Richter and Crossland 1949
1.2	Whole brain	Rat	Calculation of the rate of induced ACh depletion after an intraventricular dose of hemicholinium (20 µg)	Domino and Wilson 1972
0.15	Cerebral cortex	Cat	Calculation of the rate of ACh release from the cortex when the synthesis of releasable ACh was blocked by topically applied hemicholinium (1 µg ml^{-1})	Szerb et al. 1970
49.5	Striatum	Rat	Calculation of the *in vivo* accumulation rates of ACh following treatment with dichlorvos (15 mg kg^{-1} i.v.)	*Staviskova et al. 1976*
27	Hippocampus			
18.5	Cerebral cortex			
14.8	Medulla-pons			
7.7	Midbrain			
7.2	Thalamus			

tional properties and/or different access to AChE (Fig. 7). When animals are killed by decapitation the ACh released in the synaptic cleft at the moment of sacrifice will be rapidly destroyed by AChE in the cleft. Rapid inactivation of AChE and/or rapid tissue fixation might prevent the enzymatic hydrolysis of released ACh. Several studies indicate that newly synthesized ACh might be released preferentially (for review see MacIntosh and Collier 1976). This might explain the higher content of ^{3}H ACh after 7 s of microwave irradiation (preservation of a pool of ACh with high specific radioactivity pool b_2, Fig. 7b). The higher steady state concentration of total ACh found after 0.25 s of microwave irradiation might represent an intraneuronal pool of ACh (very low specific radioactivity pool b_1 Fig. 7a) that is destroyed by AChE unless the time of enzyme inactivation is extremely short, 0.25 s.

There is a corresponding decrease in ^{3}H-Ch content with increased content of ^{3}H ACh, but it is difficult to understand why there is no clear decrease in endogenous Ch with increased concentration of ACh.

A higher steady state concentration of ACh in rat brain was obtained by Hanin *et al.* (1970) during the light period of the day than during the dark period. Is this diurnal variation in ACh due to a change in the stable pool a or the labile pool b_1 of ACh (Fig. 7*a*)? The higher ACh values were found only in rats sacrificed by decapitation and not following freezing in liquid nitrogen (Hanin *et al.* 1970).

Endogenous ACh in the hippocampus was also found by Atweh and Kuhar (1976) to be more labile to destruction than ^{3}H ACh. Their data are, however different from the results of the present study (paper III), since they found no change in the ratio ^{3}H ACh/^{3}H-Ch in rats sacrificed by microwave irradiation of the head for 3 s (1.3 kW) in comparison with decapitation. But they obtained a higher steady state concentration of ACh after microwave irradiation (28 nmol g^{-1}) than after decapitation (22 nmol g^{-1}).

5.5 *Rate of turnover of acetylcholine in brain tissue determined by different methods of calculation*

When the specific radioactivities (SA) of a precursor and a product are plotted at different time points after intravenous administration of the precursor the time courses of the SA of the precursor and product intersect at the maximum of the product SA. According to Zilversmit (1960) this is an important criterion (but not the only one) that must be fulfilled for establishment of such a relationship. The absence of this criterion is taken as a sign of non-existance of such a relationship.

Curves indicating a precursor-product relationship between Ch and ACh have been presented for whole brain by Dross and Kewitz (1972), Sparf (1973), Jenden *et al.* (1974) and Karlén *et al* (1975) following sacrifice by decapitation and freezing in liquid nitrogen and by Cheney *et al.* (1975*b*) following microwave irradiation (2.5 kW 1 s).

Plots of the SA of Ch and ACh versus time in different brain regions with the two times of enzyme inactivation 7 s and 0.25 s are shown in Fig. 8*a*–*b* Only at the shorter inactivation time is a precursor-product relationship indicated (papers III and IV). However in the cerebellum no relationship was found. The assumption of instant

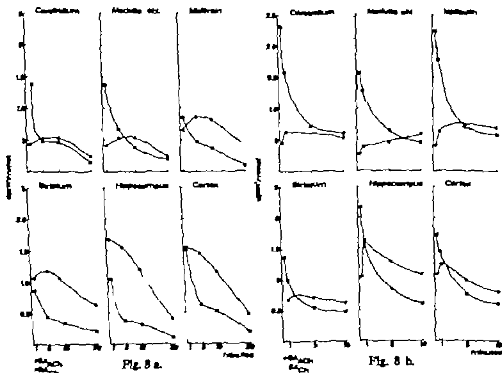


Fig. 8. The specific radioactivities of ACh and Ch are expressed as ^{3}H-ACh and ^{3}H-Ch in per cent of the total radioactivity (^{3}H tot) per nmol endogenous ACh and Ch, respectively

TABLE XI *Turnover rate of ACh (nmol g^{-1} min^{-1}) in the striatum, hippocampus and cortex calculated by five different methods from the same experimental data obtained at different time intervals after the injection of ^{3}H-Ch.*
For a description of the different methods, see text (section 5.5)

Turnover rate of ACh (nmol g^{-1} min^{-1})

Striatum	Hippocampus	Cortex	Calculation method
51	60	14	I
23	9	17	b
41	41	17	*II*
51	5	6	b
13	5	17	c
59	7	104	*III*
		14	b
55	21	27	IV
37		–	V

mixing of unlabelled and labelled Ch and subsequently a *true* precursor-product relationship may however be questioned, since endogenous ACh and ^{3}*H ACh* show different *post mortem* stabilities (3 5.3). All the same, it would be of interest to calculate an

labelled Ch can be calculated from the slope of the line obtained when plotting d (labelled ACh)/dt against $SA_{Ch}-SA_{ACh}$ for each time point (method III, Table XI). For whole brain Jenden *et al* (1974) found that two lines with different slopes could be drawn corresponding to 1 4 and 7 9 nmol g^{-1} min^{-1} (Table VIII). They concluded that these slopes represented different pools of ACh in the brain.

In the present study data from the drawn SA curves (Fig. 8*b*) at 0.25 min intervals ranging from 0.5 to 2.75 min were plotted (Fig. 10). In the striatum a line with a slope of 59 nmol g^{-1} min^{-1} was obtained (Fig. 10). In the hippocampus a linear relationship between the points was found only at the time interval 1.25–2.5 min (7 nmol g^{-1} min^{-1}). In the cortex two lines with different slopes were obtained (104 14 nmol g^{-1} min^{-1}) (line a 0.5–1.25 min line b 1.25–2.5 min).

Schuberth *et al* (1969) found that the time course of the ratio ^{3}H ACh/^{3}H-Ch increased fairly linearly up to 1 min after an intravenous pulse injection of ^{3}H-Ch. This was also the case in the brain regions studied (paper IV). Assuming that the Ch pool is known, a rough estimate of the turnover can be made (method IV Table XI).

Instead of assuming that the total free Ch pool is used for ACh synthesis, Saelens *et al.* (1974) calculated the size of the specific Ch pool from the peak value of the ratio ^{3}H ACh/^{3}H-Ch and the endogenous content of ACh. By using this theoretical Ch pool value they estimated the turnover according to Schuberth *et al* (1969). As shown in Table XI in this presentation, this method (V) could only be used for the striatum (37 nmol g^{-1} min^{-1}), since no peak value in the ratio ^{3}H-ACh/^{3}H-Ch could be found up to 10 min after ^{3}H-Ch injection in the hippocampus and cortex (paper IV).

As seen in Table XI higher turnover values are obtained when calculations are made at time points before the SA_{ACh} has reached its maximum. This is clearly seen for the hippocampus. However since the SA curves were drawn by hand, they must be approximative (experimental data at 0.5 1.5 10 min after ^{3}H Ch injection).

The values calculated in the present study by the finite difference method (method II, Table XI) are somewhat higher especially in the cortex, than those obtained by Racagni *et al* (1974) (Table IX) with the same method of calculation.

6. Effect of Sodium Pentobarbital and Oxotremorine on the Apparent Turnover of Acetylcholine in Different Brain Regions

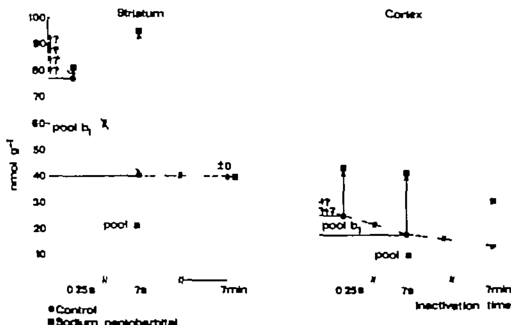
Barbiturate anesthesia increases the steady state concentration of ACh in whole brain in experimental animals (Tobias *et al.* 1946, Richter and Crossland 1949 Elliott *et al* 1950, Giarman and Pepeu 1962, paper I, Modak *et al.* 1976). It also decreases both the ACh release from the cortex (Mitchell 1966) and the apparent rate of turnover of ACh in whole brain (Schuberth *et al.* 1969 Trabucchi *et al* 1975). The ACh concentration increases in a dose-related manner with a threshold dose of 30 mg kg for sodium pentobarbital given intraperitoneally (i.p.)(paper I). The maximal increase in ACh occurs about 15 min after injection of the barbiturate (Modak *et al.* 1976). The ACh content is normal within 7 min after reappearance of the righting reflex (paper I).

In the present studies endogenous ACh was not affected to the same extent in all brain regions. Sodium pentobarbital (60 mg kg^{-1} i.p.) significantly increased the ACh content only in the midbrain, hippocampus and cerebral cortex (papers I and IV). Similar results were obtained after sacrifice by dislocation of the spine as after microwave irradiation of the head (0.25 s) (papers I and IV). However when the intermediate enzyme inactivation time (7 s) was used, a significant increase in ACh was also observed in the medulla oblongata and striatum (Nordberg and Sundwall 1975 unpublished data). In pentobarbital treated animals the ACh content was essentially the same following both types of microwave irradiation, but the values for the two groups of controls differ (data for the striatum and cortex are given in Fig. 11). Trabucchi *et al.* (1975) found that sodium pentobarbital increased the endogenous ACh in the striatum from 54 to 89 nmol·g^{-1} in rats subjected to head irradiation for 2 s (2.5 kW). The results are fairly similar to those obtained after whole body irradiation for 7 s. However Modak *et al* (1976) found also in mice treated with sodium pentobarbital and sacrificed by microwave irradiation for 0.30 s (6 kW), a significant increase of ACh from 81 to 117 nmol g in the striatum. The findings in the present studies—different control values but similar ACh values after pentobarbital treatment following both types of microwave irradiation—might indicate that pentobarbital stabilizes a very labile pool of ACh (Fig. 11). The increased ACh content in the striatum of pentobarbital treated mice following sacrifice by microwave irradiation for 0.30 s (6 kW) (Modak *et al.* 1976) is different from the finding of the present author (at inactivation time 0.25 s) and is difficult to explain.

Sodium pentobarbital decreased the apparent rate of turnover of ACh (paper IV) selectively in the hippocampus and cortex, but had no effect on the turnover in the striatum (Table XII). A similar relative effect on the turnover in the cortex and striatum was reported by Trabucchi *et al.* (1975), though their absolute values were lower

Recent studies indicate that the high affinity uptake of Ch by the cholinergic nerve terminal might be the rate-limiting and regulatory step in the synthesis of ACh (Haga

Fig. 11 EFFECT OF SODIUM PENTOBARBITAL ON ACh AT DIFFERENT TIMES OF ENZYME INACTIVATION



and Noda 1973 Barker and Mittag 1975 Mulder *et al* 1974 Simon *et al.* 1976). The latter authors found that sodium pentobarbital anesthesia significantly decreased the sodium-dependent uptake of ^{3}H-Ch in synaptosomes from cortex and hippocampus, while the uptake in striatal synaptosomes was unchanged.

The data available thus indicate that sodium pentobarbital has a selective effect on the hippocampus and cortex. A decreased release of ACh into the synaptic cleft might decrease the high affinity uptake of Ch by the neuron and reduce the synthesis of ACh. The increased steady state concentration of ACh indicates a latency between these processes.

On long-term administration of a non anesthetic dose of barbital to rats in the drinking fluid (250 mg kg^{-1} day^{-1}) for 30 weeks no effect was seen on the brain steady state concentration of ACh when the barbital was administered up to the time of decapitation (paper VI). When the drug was withheld for 3 days a significant decrease in ACh was found only in the striatum. On the 12th day of such an abstinence a decrease in ACh was found both in the striatum and brain stem + cerebellum. No effect was found in the cortex + hippocampus at either of the two time points of the abstinence. The findings are in contrast to the effect of a single anesthetic dose of sodium pentobarbital.

Like pentobarbital, the muscarinic agonist *oxotremorine* increases the steady state concentration of ACh in the brain (Holmstedt *et al* 1963 Cox and Potkonjak 1969 Sethy *et al.* 1973) and decreases the apparent turnover of ACh (Schuberth *et al.* 1969

TABLE XII *Effect of sodium pentobarbital (60 mg kg^{-1} i.p.) and oxotremorine (1 mg kg^{-1} i.p.) on the apparent rate of turnover (nmol g^{-1} min^{-1}) of ACh in different mouse brain regions*

Brain region	Control	Sodium pentobarbital	Oxotremorine
Cerebellum	(2)	(2)	–
Medulla oblongata	10	7	–
Midbrain	11	9	5
Striatum	55	59	44
Hippocampus	21	6	7
Cortex	27	10	8

Trabucchi *et al* 1975). In contrast to pentobarbital it also increases the steady state concentration of endogenous Ch in the brain (Schuberth *et al.* 1969 Ladinsky *et al* 1974 Trabucchi *et al.* 1975). Szerb and Somogyi (1973) found that oxotremorine *in vitro* decreased the release of ACh from the cerebral cortex.

Oxotremorine selectively increases the endogenous ACh concentration in the cortex, hippocampus and striatum while no effect is found in the midbrain, medulla oblongata and cerebellum (Campbell and Jenden 1970, Choi *et al* 1973 papers II, VII). In the striatum, however its effect on ACh is less pronounced in animals sacrificed by microwave irradiation than in decapitated animals (Cheney *et al.* 1976, Karlén *et al* 1976, papers II VII).

The effect of oxotremorine on the biosynthesis of ^{3}H ACh in the striatum was also found to differ following the two methods of sacrifice (papers II, VII Karlén *et al.* 1976). Following dislocation of the spine the biosynthesis of ^{3}H ACh was increased and the effect could not be counteracted by pretreatment with atropine (paper II). Following microwave irradiation the biosynthesis was decreased and the effect was counteracted by pretreatment with atropine (paper VII). Oxotremorine decreased the ^{3}H ACh biosynthesis in the other brain regions following both methods of sacrifice, and the effect was prevented by pretreatment with atropine (papers II, VII).

A possible reason for these different effects on both ^{3}H ACh and endogenous ACh in the striatum is that oxotremorine might have a stabilizing effect on the labile ACh pool(s) which is/are destroyed by AChE in untreated animals unless the enzyme is not instantly inactivated by microwave irradiation.

Increased values of endogenous Ch have been obtained in mouse brain regions following oxotremorine treatment (Karlén *et al.* 1976, paper VII). As discussed in paper VII this might be due to a combined central and peripheral effect (hemodynamic effect, see below). A solely peripheral effect is less likely due to the finding of Lundgren *et al.* (1976) that pretreatment with methylatropine prevented the increase of Ch in plasma but not in brain.

In study VII oxotremorine decreased the apparent rate of turnover of ACh in the cortex, hippocampus, midbrain and striatum (the only brain regions studied in this respect) (Table XII). This is in agreement with the decreased ACh turnover in the lower brain stem, diencephalon, striatum and cortex found by Choi *et al.* (1973) following decapitation and in the striatum observed by Karlén *et al.* (1976) following microwave irradiation.

Simon *et al* (1976) reported a decreased *in vitro* uptake of ^{3}H-Ch in synaptosomes from hippocampus of oxotremorine treated rats. Preliminary observations by the present author indicate that the uptake is also decreased in the cortex and striatum (Nordberg 1977 unpublished observations). The effect of oxotremorine thus seems to be different from the effect of pentobarbital, where no effect was found on the ^{3}H-Ch uptake in striatal synaptosomes. A stimulating effect of oxotremorine on the ACh receptors might lead to a diminished release of ACh into the synaptic cleft, a reduced neuronal high affinity uptake of Ch and a reduced synthesis of ACh in the neuron.

The possibility may not be overlooked that drug treatment might influence the kinetics of the injected radioactive Ch. After treatment with sodium pentobarbital (60 mg kg^{-1}), the initial brain uptake of intravenously administered ^{3}H Ch and the brain concentration of unmetabolized ^{3}H-Ch were lower (by 20—40%) than in the controls (paper V). A correspondingly (40%) lower concentration of unmetabolized H-Ch was also found in the plasma of pentobarbital treated animals (paper V). These findings indicate that sodium pentobarbital might, by hemodynamic effects, influence the kinetics of a tracer dose of ^{3}H-Ch.

Oxotremorine (1 mg kg^{-1}), on the other hand, increased the plasma concentration of ^{3}H-Ch (by 70%) as well as the initial brain uptake of ^{3}H-Ch (by 50%) and the brain concentration of unmetabolized ^{3}H-Ch (by 80%) (papers II V). Karlén *et al* (1971) found a higher content of oxotremorine in the plasma and brain of rats treated with oxotremorine alone than in those treated with methylatropine and oxotremorine combined, and concluded that oxotremorine might decrease its volume of distribution. Higher plasma values of intravenously injected radioactive dextran (^{3}H-dextran) were also found in oxotremorine treated mice (paper II). It therefore seems plausible that the effect of oxotremorine on the brain ^{3}H-Ch content may be explained by hemodynamic changes. Higher steady state concentrations of Ch have also been found in the plasma and brain in rats treated with oxotremorine (Karlén *et al* 1976 Schuberth *et al* 1969 Ladinsky *et al* 1974). The increase in the brain content of endogenous Ch caused by oxotremorine might partly be due to peripheral (hemodynamic) effects (Lundgren *et al.* 1976, paper VII).

When the apparent turnover of ACh was calculated in studies IV and VII no compensation was made for extracerebral radioactivity in the blood remaining in the brain tissue (Aquilonius and Eckernäs 1976). Thus, the hemodynamic effects of pentobarbital and oxotremorine might alter the calculated turnover values of ACh. In study II H-dextran was given intravenously and from the data obtained, the volume of the blood remaining in the cortex is calculated to be 20 µl g. Taking into account the content of unconverted H-Ch in remaining blood of the brain, the turnover in the cortex is calculated to be 35 nmol g in control animals and 11 nmol g in oxotremorine treated animals. The turnover values are thus a little higher than those obtained without compensation for extracerebral ^{3}H-Ch (26 nmol g and 8 nmol g respectively paper VII). However a 70% decrease in the rate of turnover of ACh is found to follow oxotremorine treatment with both methods of calculation

The importance of the time of enzyme inactivation for the steady state concentrations of ACh and Ch, and the biotransformation of a tracer dose of ^{3}H-Ch have been systematically investigated in six well-defined parts of the mouse brain. To get a shorter time of enzyme inactivation than with conventional killing two methods of microwave exposure were used. Whole body irradiation for 7 s (1.3 kW) or head irradiation for 0.25 s (5 kW) increased the brain temperature to 85–90°C. The time courses of the temperature after completion of irradiation were similar

A markedly higher content of ^{3}H ACh was preserved, especially in the striatum, when the time of enzyme inactivation was reduced from 7 min (decapitation) to 7 s. A comparable increase in the steady state concentration of ACh was obtained on reducing the time of inactivation further to 0.25 s. The findings indicate the existence of several pools of ACh in the brain

a. A fairly stable pool which is not changed during the time interval of some seconds to some minutes after sacrifice.

b_1 A very labile pool which is destroyed by AChE unless the time of enzyme inactivation is extremely short. This pool constitutes about half the total ACh content in the striatum.

b_2 A pool of newly synthesized (^{3}H ACh) which is more stable than pool b_1. This pool is also easily demonstrated in the striatum.

Although these studies indicate that ACh in a brain region is not homogeneous, plots of the specific radioactivities (SA) of ACh and Ch at different time points after injection of ^{3}H-Ch indicated a precursor-product relationship in all brain regions (except in the cerebellum) at the shorter time of enzyme inactivation (0.25 s).

The data obtained were applied to different models of turnover calculations. The calculated turnover rate values must be considered rather approximate (apparent) owing to our lack of knowledge of the size of the Ch pool available for ACh synthesis. Higher values were found when the calculations were made at time points before SA_{ACh} had reached its maximum. The turnover values differed between the brain regions and the highest rate of apparent turnover was found in the striatum (55 nmol g min). In the cortex and hippocampus this value was approximately half and in the midbrain and medulla oblongata approximately a fifth.

Sodium pentobarbital and oxotremorine increased the steady state concentrations of ACh selectively in the brain. A marked increase in ACh was found in the cortex and hippocampus after treatment with both drugs. Oxotremorine also increased the content of ACh in the striatum. A plot of the SA of ACh and Ch indicated a precursor-product relationship in the brain regions also for drug treated animals. This motivated a calculation of an apparent turnover rate of ACh. Sodium pentobarbital decreased markedly

apparent rate of turnover of ACh only in the cortex and hippocampus. The effect of oxotremorine was less selective and a decreased rate of turnover was obtained in all four brain regions studied in this respect.

Pentobarbital and oxotremorine treatment appeared to have a stabilizing effect on the labile ACh pools (b_1 and b_2).

In rats treated with barbital for 30 weeks and the barbital was withheld for 3 days a decrease in the steady state concentration of endogenous ACh was obtained specifically in the striatum while no change was found in the cortex + hippocampus.

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ACTA PHYSIOLOGICA SCANDINAVICA

Supplementum 446

OBSERVATIONS ON THE MORPHOLOGY AT THE TRANSITION BETWEEN THE PERIPHERAL AND THE CENTRAL NERVOUS SYSTEM IN THE CAT

T Carlstedt

I. A preparative procedure useful for electron microscopy of the lumbosacral dorsal rootlets.

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II. General organization of the transitional region in S_1 dorsal rootlets.

C. H Berthold and T Carlstedt

III Myelinated fibres in S_1 dorsal rootlets.

T Carlstedt

IV Unmyelinated fibres in S_1 dorsal rootlets.

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V A light microscopical and histochemical study of S_1 dorsal rootlets in developing kittens.

STOCKHOLM 1977

MATERIALS METHODS AND EVALUATION NORMS

Twenty-three adult cats (see Fig. 1) were anaesthetized intraperitoneally with pentobarbital, 40 mg/kg BW and artificially ventilated with 100% oxygen. Th lower spinal cord and its roots were fixed by vascular perfusion through the thoracic aorta, using a controlled intermittent pressure which varied from 0 up to 70-100 mm Hg (see Berthold, 1968 a). After a laminectomy the lower lumbar and the first sacral cord segments together with their dorsal roots were removed. Each root was carefully separated with the aid of watchmarker s forceps along the cleveage planes offered by the rootlets. After trimming with a razor blade the specimens consisted of the proximal on third of the rootlet and its attachment to the spinal cord with a small piece f the surrounding white matter

In the case of immersion fixation (see Fig 1) the specimens were removed from the animal after a laminectomy carfully placed on strips of filter paper and then immediately put in the various ice-cold fixatives (see below) The tonicities of all solutions were tested on a Knauer osmometer

For light-microscopic survey pictures, specimens obtained after the perfusion fixation were frozen, longitudinally sectioned into 15-20 µm thick slices in a cryostate at -30°C and stained in alkaline toluidine blue (1% toluidine blue in a 1% solution of $NaHCO_3$,pH8) heated on the glass lide

Preparative experiments

1 *Th original prepa t e method (f Berthold, 1968 a, 3 cat see Fig 1)* The perfusate consisted f 5 glutaraldehyde in a 300 mOsm phosphate buffer pH 7.2 7.4 prepared according to Millonig (1.8 g NaH PO H O 23.2) g Na HPO $7H_2O$ and 5 g NaCl dissolved in 1000 ml f distilled water see Karlsson and Schultz, 1965) containing 2.7% Dextran T70 (Pharmacia, Uppsala, Sweden) (cf Berthold 1968 a, Bohman and Maunsbach 1970). After a postfixation at 4°C for 4 hours in a solution of the same composition as the perfusate the were rinsed overnight at 4°C in a 300 mOsm Millonig buffer osmificated for 4 urs at 4 C in 2% OsO 300 mOsm Millonig buffer nd then again rinsed for 0.5 hours at 4 C in a 300 mOsm Millonig buffer The specimens were dehydrated at room temperature (cf. Sterlz g and Napol tano, 1971) in 50 70 80 90 and 95% acetone water mixtures (one hour in each concentration) and in absolute acetone (two hours) and were embedded in Vestopal W (Ryter and Kellenberger 1958). I filtration wa performed in 25, 50 and 75% Vestopal acetone mixtures for 3-4 hours in each step and n 100% Vestopal overnight at room temperature Polymerization took place first at 4 C for 24 hours and then at 60°C for 48 hours. Each of the arious steps in the preparative procedure given above wa submitted in consecutive order to a set of tests A schedule of the rious experiments is given in Fig 1

Immersion fixation (3 cats see Fig 1 and T ble I) 5 glutarald hyde was used. The fixati e vehicle consisted of the M llonig buffer (100-500 mOsm) with the addition f sucrose (0-500 mOsm) or NaCl (0-500 mOsm)

3. *Perfusion fixation (8 cat see Fig 1 nd T ble II)* The perfusates tested consisted of 5% glutaraldehyde in a 300 mOsm Millonig buffer containing 7% dextran. Sucrose (0-250 mOsm) and or NaCl (0 300 mOsm) were added t d fferent concentrations.

4 *Rinsing and osmication (4 cats see F g 1 and T ble III)* Specimens obtained after perfusion fixation % glutaraldehyde in a 300 mOsm M llonig buffer containing 2.7% dextran and 200 mOsm sucrose were rinsed i 300 mOsm M llonig buffer and osmicated in 2% OsO dissol ed in 300 mOsm Millonig buffer Sucrose (0-400 mOsm) w added in different concentration *to the rinsing solution used after glutaraldehyd fixation and to the osmium* solution.

5 *Dehydratio (6 cat see F g 1 nd Tabl IV)* Specimens perf sion fixed a described under (1), rinsed in 200 mOsm M llonig buffer with the addition of 200 mOsm ucrose and osmificated in 2% OsO in 300 mOsm M llonig buffer were dehydrated in acetone The dehydration procedure was aried with respect to dehydration time n mber f dehydration steps and the concentration of aceton the d fferent teps In one case the pecimens were d hydrated conti uou ly from 0 to absolute acetone for eight hours

6. *Embedding (3 cats, see Fig. 1)*: Specimens processed as described in section 5 and submitted to a stepwise and prolonged dehydration (N in Table V) were embedded in Vestopal W or Araldite (Glauert *et al.*, 1956) or Epon (Luft, 1961) or Spurr (Spurr, 1969). Specimens intended for embedding in Araldite or Epon were dehydrated in ethanol either in a prolonged and stepwise way (N in Table V) or with only few steps (cf. Luft, 1961) and after dehydration soaked for 0.5 hours in propylenoxide. Infiltration in the different resins took place in 25, 50 and 75% solutions for 3-4 hours and in 100% resin overnight at room temperature. Polymerization of Araldite, Epon and Spurr was performed for 3 days and at 60°C.

7. *Additional experiments (4 cats)*: These experiments were all minor modifications of the final procedure given in Table I.

(a) Digitonin (Sigma, USA) was added to a concentration of 0.2% to the glutaraldehyde-postfixative solution and the subsequent rinsing solution (cf. Napolitano and Scallen, 1969).

(b) The perfusate contained 4% glutaraldehyde and 1% formaldehyde instead of 5% glutaraldehyde as fixative agent. The postfixative solution was of the same composition as the perfusate.

(c) Ethanol was used instead of acetone as a dehydration agent (Vestopal embedding). The specimens were then rinsed in styrene and infiltrated in a 1:1 styrene-Vestopal mixture under a moderate vacuum according to Kurtz (1961).

(d) The water-miscible embedding medium Reform I (resorcinol formaldehyde resin) (Robertson and Parsons, 1970; Hildebrand, 1974) and glycol methacrylate (Rosenberg *et al.*, 1960; Leduc and Berhard, 1967) were used instead of dehydration in acetone and embedding in Vestopal. Before embedding the specimens were cut into 0.1 mm-thick transverse slices on a Smith-Farquhar TC-2 tissue sectioner (Sorvall, USA). The slices intended for embedding in Reform I were stained *en bloc* in a solution of 1% uranyl acetate in 300 mOsm Veronal-acetate buffer for 1 hour (for details see Hildebrand, 1974).

The length of six roots were measured in between each step of the preparative sequence of the useful methods (see Fig. 1) using a preparatory microscope with fixed scale. This was done in order to determine the degree of shrinkage.

Preparative steps performed after polymerization

These steps remained unchanged throughout the investigation. The specimen blocks were trimmed to a pyramid using a high-speed dental burr and razor blades and sectioned on a LKB 4800 Ultrotome. Semithin sections (0.5 μm thick) were placed on glass slides and stained with alkaline toluidine blue. The sections were examined and photographed in a Leitz photomicroscope (Ortolux).

Ultrathin sections (the interference colour of which varied from gray to white-yellow corresponding to a thickness of approximately 500-1000 Å cf. Peachey, 1958) — were placed on one-hole copper grids (Veco, Germany) coated with a carbon-stabilized formvar film. After double staining with uranyl acetate (Brody, 1959) and lead citrate (Reynolds, 1963) the sections were examined in a Philips EM 300 electron microscope operated at 80 kV and equipped with a 30 μm objective aperture.

Evaluation norms

The current criteria for an acceptable ultrastructural preservation of a tissue in general (see *inter alia* Palade, 1952; Maunsbach, 1966 a; Sjöstrand, 1967; Millonig and Marinozzi, 1968) and of nervous tissue in particular (*among others see* Webster and Spiro, 1960; Elfvin, 1961; Williams and Landon, 1963; Landon and Williams, 1963; Webster and Collins, 1964; Karlsson and Schultz, 1965; Johnston and Roots, 1967; Berthold, 1968 a, b; Hildebrand, 1971; Schultz and Karlsson, 1972; Karlsson *et al.*, 1975; Kalimo, 1976) were adopted. Thus the tissue of the transitional region should appear free from empty torn spaces, myelin sheath splitting, defects in cellmembranes and disarranged cytoplasmic constituents if its preservation was to be considered as useful.

Brief comments on the methods

Glutaraldehyde was used in view of its well documented superiority as an ultrastructural fixative in general (Sabatini *et al.*, 1963; Maunsbach, 1966 a, Millonig and Marinozzi 1968; Hopwood, 1969 1972) and in particular with regard to nervous tissue (Karlsson and Schultz, 1965; Berthold 1968 a; Conradi, 1969, Hildebrand, 1971). The concentration of the glutaraldehyde was kept at 5% in all but one experiment (7:b) in order to avoid a poor tissue penetration when using a too low concentration (Hopwood, 1969) and a hypertonic effect of too high a concentration (cf. Berthold, 1968 a; Hildebrand, 1971). Likewise because of already demonstrated advantageous preparative effects, the phosphate buffer containing 0.5% NaCl (Millonig 1961 Maunsbach, 1966 Millonig and Marinozzi 1966) and 2.7% low molecular dextran (Berthold, 1968a, Bohman and Maunsbach, 1970) as well as the method of administrating the fixative by vascular perfusion (Palay *et al.*, 1962; Maunsbach, 1966 a) were without further testing adopted from the so-called »original preparative method (Berthold, 1968 a). Immersion fixation was used when studying the effects of different vehicular tonicities.

The results of the experiments were evaluated for each set f tests by examining two or more specimens from each cat. The modification within a test group that rendered the least artifactitious appearance was classified as the »relatively best» method and was selected for further experiments now in combination with various modifications of the consecutive step in the preparative sequence. In this way a final series of modified preparative steps was obtained, which when taken together constituted what was referred to as the useful method» (see Fig 1).

RESULTS

General description of the transitional region

A cross section through the transitional region reveals two concentric compartments separated at the PNS–CNS borderline, an outer PNS compartment and an inner CNS compartment (Figs. 2, 3 4). The CNS compartment discloses an *inner »core zone» showing the arrangements of a central fibre tract and an outer covering* mantle zone» consisting of astrocytes and nerve fibres (Figs 3 4). The transition of a myelinated nerve fibre from the peripheral to the central nervous type (Figs 5 and 6) occurs in the mantle zone at a node of Ranvier the borderline node (for a full description and definition of terms see Berthold and Carlstedt, 1977).

Preparative experiments

Original preparative method (Exp 1 Figs 1 4 5). As shown in Figs 4 and 5 the apperance of the PNS compartment of the transitional region corresponded to that of a successfully performed preparation.

In the CNS compartment and particularly in the mantle zone the appearance of the tissue was far from satisfactory The myelin sheaths of the large and medium sized fibres (4-5 µm in diameter) and of most small fibres revealed splitting, which was most severe close to the borderline nodes (i.e at the termination of the central internode) Moreover there occurred comparatively large empty spaces both outside and inside the myelin sheaths (Fig. 4). Empty spaces and disarranged astroglia were also noted at the borderline nodes of Ranvier The node gaps appeared as if they had been torn open and they contained randomly arranged Schwann cell and glial processes (Fig 5)

Modifications of the original steps in the preparatory procedure (Exp. 2 3 4 5 6): The progression of this step-by-step development is given in Fig. 1 The preservation quality of the PNS-compartment was in all experiments superior to that of the CNS-compartment. The artifacts noted in the different experiments (2-6) all belonged to the same general pattern of disarrangement as disclosed by the TR after treatment according to the »original preparative method and represented exaggerations or mitigations of this very pattern.

Immersion fixation (Figs. 6-14, Table 1) in a normotonic or slightly hypertonic Millonig buffer (Figs. 8, 10 11 13) gave the »relatively best« results of experiment group 2. Immersion fixation as such seemed to exaggerate the disarrangements (compare Figs. 6 and 8 with Figs. 4 and 5). Treatment with equiosmolar 5% glutaraldehyde solutions containing either NaCl or sucrose gave distinctly different preservation patterns when the tonicites of the added substances exceeded 300 mOsm (compare Figs. 12 and 14).

The next two sets of experiments: perfusion fixation (exp. 3: Table II Figs. 15-18) and rinsing and osmication (exp. 4. Table III, Figs. 19 20) showed that the addition of 200 mOsm sucrose to the perfusate and to the rinsing solution gave the »relatively best« results respectively. An addition of more than 200 mOsm sucrose to the rinsing solution gave rise to a serious and generalized splitting of all central myelin sheaths (Fig 19).

The testing of different modes of dehydration (Table IV Figs. 21 26) showed that a procedure that started in 10% acetone and progressed in 10%-steps for 1-hours intervals to 100% aceton rendered the »relatively best« preservation. As compared to Vestopal W (Fig. 26) no improvements were obtained by embedding in Epon, in Araldite (Fig 27), or in Spurr (Fig 28). The latter two media gave rise to a considerable disarrangement of the CNS-compartment.

To sum up, a degree of preservation classified as »useful« was obtained by vascular perfusion with 5% glutaraldehyde in 300 mOsm Millonig buffer with the addition of 200 mOsm sucrose and 2.7% low molecular dextran, postfixation in the same medium for 4 hours; a rinse overnight in 300 mOsm Millonig buffer with the addition of 200 mOsm sucrose; osmification in 2% OsO_4 in 300 mOsm Millonig buffer a slow and extensive stepwise dehydration in acetone and embedding in Vestopal W. In specimens treated according to this procedure, the myelinated nerve fibres of the core zone appeared almost free of both myelin splitting and tissue defects in the adjoining glia. Small discrete and solitary myelin fissures and splittings were present in the mantle zone and large myelin sheaths appeared somewhat wavy or serrated (Figs. 3, 26). Borderline nodes were closely surrounded by astrocytic elements, contained Schwann and glial processes and only a few small torn, empty-looking defects were noted. Small fibres constantly revealed a better preservation than did larger ones.

The longitudinal shrinkage of the specimens was about 10% (Table V).

Additional experiments (exp 7): In this set of experiments the »useful« method was submitted to minor modifications.

The addition of digitonin (7:a) to the fixative and rinsing solutions (Fig 29) gave well preserved myelin sheaths but introduced serious disarrangements of glial, Schwann cell and axon membranes. Neither the use of a formaldehyde-glutaraldehyde fixative (7:b Fig 30) nor dehydration in ethanol prior to Vestopal embedding (7:c; Fig. 31) improved the preservation. Embeddings in watersoluble media (7:d) were likewise without success. GMA (Fig 32) provoked severe myelin splitting and glial shrinkage. Reform I (Fig. 33) gave a very weak positive contrast after staining *en bloc* in uranyl acetate. A »negative« contrast pattern appeared after section staining with lead. Here myelin sheaths were easily identified as light rings on a black background (Fig. 33). Glial cells, Schwann cell and axons however all required high magnification in order to be visualized. Myelin sheath splitting was minute. The sections showed a strong tendency to disintegrate when exposed to the electron beam.

DISCUSSION

The present study has shown that certain modifications of the »original preparative method» did improve the ultrastructural appearance of the transitional region — notonous for the difficulties involved in its preservation (cf. Foncin, 1961 Maxwell, 1967 Maxwell *et al.* 1969· Ross and Burkel, 1971 Steer 1971). Considerable improvements were noted with regard to the following very troublesome artifacts, which all seemed to be aggravated as fibre size increased. loss of paranodal CNS-myelin sheath compactness high occurrence of CNS-myelin sheath fissures large empty spaces outside and inside CNS-myelin sheaths empty spaces between glial processes and torn and disrupted nodes of Ranvier Nevertheless, minor defects presumably induced by myelin lipid extraction and the high osmotic pressure of the »useful method (see below) still remained. These artifacts appeared in the form of tiny spaces outside central nervous myelin sheaths, as also did small fissures in the terminal (close to node) segments of the largest myelin sheaths and a more or less corrugated appearance of CNS-myelin sheaths These aberrations being more pronounced in larger fibres and particularly in the mantle zone were less pronounced in the core zone and were quite rare in the PNS compartment.

A comparison between the performance of the individual steps of the »original preparative method» and that of the useful one shows that the improvements of the preservation is connected with the following steps (a) the addition of 200 mOsm sucrose to the fixative (b) the addition of 200 mOsm sucrose to the rinsing solution after glutaraldehyde fixation, and (c) the use of a prolonged and extensive stepwise dehydration. The present study also added much support to the view that vascular perfusion fixation is superior to immersion fixation (cf Figs. 5 and 6).

It is well known that the tonicity of a fixation solution is highly significant as regards the tissue preservation obtained (Palade 1952; Robertson 1964 Holt and Hicks 1961 Elfvin 1962; Karlsson and Schultz, 1965; Maunsbach, 1966 b; Millonig and Marinozzi, 1968, Berthold 1968 a, Hildebrand, 1971). With regard to myelinated nerve fibres the use of isotonic or slightly hypertonic vehicles has proved to give the best results (Webster and Collins 1964 Schultz and Karlsson, 1965· Berthold 1968 a, Hildebrand, 1971). Usually NaCl and/or sucrose are used to regulate the osmotic pressure (Holt and Hicks 1961 Maunsbach, 1966 b· Brunk and Eriksson, 1972)

The total osmotic pressure of the fixative employed in the useful method is theoretically close to 1000 mOsm. In terms of effective osmotic pressure — some 60–100% of the theoretical tonicity of the fixing agent, here containing 5% glutaraldehyde is considered to be osmotically ineffective (cf Schultz and Karlsson, 1965; Maunsbach, 1966 b· Bone and Denton, 1971 Brunk and Eriksson, 1972; Penttila *et al.* 1974) — this should correspond to 500-700 mOsm, i.e. a pressure about twice that of serum. The observation that such a comparatively high total tonicity renders a useful preservation of the CNS compartment of the transitional region is nevertheless in full agreement with the data of Hildebrand (1971) on feline spinal cord white matter Thus Hildebrand added 150-200 mOsm NaCl or 200 mOsm sucrose to the 300 mOsm Millonig buffer In the present study however sucrose was found to be superior to NaCl as osmotically active agent. The choice of the former substance was made because although quite comparable to NaCl with regard to the better preservation of the core zone it consistently gave a somewhat better preservation of the mantle zone characterized by fewer myelin fissures, fewer glial defects and less torn node gaps (Figs. 15 and 18)

The rationale behind the differentiated influence of equiosmolar additions of sucrose and NaCl to 5% glutaraldehyde in a standard vehicle as shown in Figs 12 and 14 is obscure. In this context there are however some sparse data of interest which have mostly been derived from fixation model systems such as pure protein solutions, tissue cultured cells and sea urchin eggs (see Millonig and Marinozzi,

1968, Penttilä *et al.* 1974). Unfortunately all these are far from possessing the complexity displayed by the highly intricate system of densely packed and interwoven high-lipid low water (myelin) and high-water low-lipid (cytoplasm and endoneurial space) elements that constitute the transitional region.

In the first place NaCl dissociates into charged particles and sucrose does not. The great significance of the ionic strength with regard to the stability of the myelin sheath has been demonstrated by Robertson (1964), who showed that the severe myelin sheath splitting evoked by osmotic shock in distilled water could not be reversed by pure osmotic forces (sucrose) but responded promptly to a change in ionic strength (see also Worthington and Blaurock 1969) Since the 300 mOsm Millonig buffer contains some 100 mOsm NaCl and the advantageous effects of sucrose addition were noted at a tonicity of 100-200 mOsm, the possibility exists that an osmolarity ratio of 0.5-1 between NaCl and sucrose is optimal with regard to myelin sheath stability during fixation of the mantle zone. Moreover as shown by Millonig and Marinozzi (1968) NaCl and sucrose are strikingly different in their capacity to catalyse the »fixation reaction» as estimated from »gelling time» The former substance was by far the more active one. The membrane protective action of sucrose is well known and is in fact exploited in most EM histochemical procedures (cf. Brunk and Eriksson, 1972)

From the observation of a low content of vessels in the transitional region (Berthold and Carlstedt, 1977) it might be expected that the rapid penetrating abilities of formaldehyde would be advantageous when using a formaldehyde-glutaraldehyde mixture as fixative (cf, Karnowsky 1965). However no improvement in preservation of the tissue was noted with this modification (Fig. 30) This might be due to the fact that formaldehyde, although it rapidly penetrates a tissue, is a slow-acting fixative the effects of which are reversible (Millonig and Marinozzi, 1968, Hopwood, 1977).

The dehydration step means substitution of an anhydrous organic solvent for water as well as a considerable extraction of lipids such as cholesterol, cholesterol esters triglycerides and free fatty acids (Adams and Bayliss, 1968). During dehydration the interlamellar distance of myelin decreases by about 30% (from 180 Å to 120 Å) and about 40-50% (from 160 Å to 110-80 Å) in the PNS and the CNS respectively (Finean 1953, 1960 a,b Fernandez-Moran and Finean, 1957 Moretz *et al.*, 1969 a,b; Hildebrand and Müller 1974) Thus the whole myelin sheath shrinks some 30-50% in the radial direction but as found here (Table V) only about 10% in the longitudinal direction. This is a situation where mechanical stresses and shearing forces are apt to develop at those sites where the ordered high-lipid low water compartment of compact myelin is interfered by the low-lipid high-water compartments of cytoplasm and extracellular space, i.e. at the interfaces between a sheath and its surroundings. The incisures of Schmidt-Lanterman and the nodes of Ranvier are particularly noteworthy here, since at these sites tiny compartments of cytoplasm are more or less secluded in the compact myelin.

The use of a slow and stepwise dehydration that started at 10% acetone and included passage through several concentration steps below 50% proved to be the modification of the »original» used preparative procedure that yielded the comparatively greatest improvement. Conceivably a slow progression from low to high concentrations would induce less drastic fluid fluxes and less stress in the tissue than would a procedure starting at 50% acetone.

Accounting for the facts that lipid loss and radial myelin sheath shrinkage are more or less restricted to the highest acetone concentrations (Mühlentaler 1958, Sterning and Napolitano 1971) and that these higher concentrations were used both in the »original preparative» and in the »useful» method, it seems probable that the lower acetone concentrations of the adopted dehydration schedule might actually have increased the stability of in particular the myelin sheaths.

This recalls the earlier use of acetone as a primary fixing agent in light microscopy (Pearse, 1961) as well as in the leather tanning industry in that case in combination

with aldehydes (Gustavsson 1956) Although the effects of dehydrating agents as a rule are treated in terms of mere water substitution their possible role as post fixatives has been discussed by among others, Johnston and Roots (1967) and Schultz and Karlsson (1972) In line with those workers and taking into account the present results, it seems reasonable to ascribe some sort of myelin sheath post fixation property to the acetone as used here.

Embedding in water miscible media like glycol methacrylate and resorcinol formaldehyde whereby dehydration is avoided, were not successful. Neither were there noted any further improvements by the addition of digitonin — a cholesterol-protective agent (Napolitano and Scallen 1969 Sterzing and Napolitano 1971) — to the fixative nor through the use of ethanol as a dehydrating agent, a procedure claimed to diminish lipid extraction (Sterzing and Napolitano 1971) or through the embedding of specimens that had been acetone dehydrated to 90-95% In full agreement with Condie *et al.*, (1961) Vestopal W appeared to be a highly reliable nerve fibre embedding medium.

The best preservation was always noted in the PNS-compartment where the myelinated fibres constituted individual units distributed in a large more or less empty-looking endoneural space. A less satisfactory preservation was obtained in the core zone of the CNS-compartment where the fibres are densely packed, often with thin rim-like demarcations of glial cytoplasm between them The least satisfactory results were constantly found in the mantle zone where the paranodal segments of the central fibres are found surrounded by comparatively large areas of astrocytic cytoplasm

Two facts emerge when one looks for the rationale behind the differencies noted in the preservation of the PNS and CNS compartments. (a) the CNS-compartment lacks blood vessels (see Berthold and Carlstedt, 1977) (b) there are differences in the chemical composition of PNS and CNS myelin (Evans and Finean, 1965 Wolfgram and Kotorii, 1968 a,b). The ultrastructural preservation of a vulnerable tissue compartment that lacks blood supply undoubtedly offers great problems and it might be asked whether vascular perfusion really is at all meaningful for the fixation of the transitional region. However the comparatively high occurrence of blood vessels in the PNS compartment and their distribution along the PNS-CNS borderline (Berthold and Carlstedt, 1977) speak in favour of a fixation administrated via the vascular route This is supported by the successful use of perfusion and the complete failure to obtain acceptable preservation by immersion fixation in the present work.

PNS and CNS myelin are known to differ with regard to both proteins and lipids. (Finean 1960 a Hildebrand and Müller 1974). X-ray diffraction analysis has shown that the interlamellar myelin sheath period of unfixed and of fixed, dehydrated and embedded PNS and CNS specimens is 180 Å and 120 Å and 160 Å and 80-110 Å respectively (Evans and Finean, 1974 Wolfgram and Kontorii, 1968 a,b). In the case of a myelin sheath of 130 lamellae (fiber size 15-20 μm) this differentiated shrinkage on the two sides of the borderline node implies that the radial shrinkage on the CNS side is about 1 6 times that on the PNS side. This gives in absolute figures a fibre shrinkage of more than 2 μm in the CNS as compared with 1.2 μm in the PNS. Consequently the CNS fibres should be more exposed than the PNS fibres to mechanical stress and shearing forces during preparation.

While it seems possible to argue that the general differences in PNS-fibre ultra structural preservation as compared with that of CNS-fibres are likely to be due to differences in the chemical composition of the myelin this explanation is hard to adopt with regard to the differences noted between the mantle- and the core-zones. These two regions are occupied by the same internodes and there are no data available that support the idea that the fundamental myelin sheath composition varies along one and the same internode. On the other hand, the CNS paranodal region (cf Hildebrand, 1971) might well in analogy with PNS paranodes, be a particularly vulnerable fibre region. However since distorsion of CNS-paranodes, as seen in feline spinal cord white matter (Hildebrand, 1971) can be considerably

reduced by the use of a fixative solution that has been made [illegible] addition of either 100-200 mOsm NaCl or sucrose and that ad[illegible] little effect as regarded the CNS borderline paranodes — [illegible] effective agent — other factors seem to influence the preservation of [illegible] myelin. One such additional factor could be the position of the [illegible] interface between two tissue compartments with different shrinkage [illegible]

Why then does not the PNS borderline paranode become dis[illegible] to the borderline as the CNS paranode. This brings up the question of [illegible] arrangement of the nerve fibres in the PNS compartments and [illegible] pointed out above, the myelinated fibres are, in the PNS, totally [illegible] neighbour fibres by the endoneural space; a connective tissue [illegible] containing some bundles of collagen fibres a few fibroblasts and [illegible] structureless ground substance. In the mantle zone the interspace [illegible] myelinated fibres is completely occupied by a mosaic of [illegible] processes. Hence, there is a superficial resemblance between the [illegible] of high-lipid, low water material are suspended in a [illegible] environment. In the PNS, however each tube appears to be [illegible] change shape within rather wide limits without seriously dis[illegible] The structureless extracellular space of the endoneural compartment [illegible] prevents the tracing of such changes in the electron microscope [illegible] on the other hand, the intervening space is in principle [illegible] exposed to osmotic influence. It is further supplied by all [illegible] well as by a number of specific structural proteins. Concen[illegible] shrinkage in the mantle zone should then give rise to the form[illegible] torn components and splitting

To sum up, it can be suggested that the difficulties met with [illegible] of the transitional region for electron microscopy should [illegible] factors; a difference in the chemical properties of CNS and P[illegible] rise to a sharp shrinkage gradient in the PNS-CNS borderline [illegible] of the mantle zone. The formation of torn empty-looking spa[illegible] splitting were to a considerable extent prevented by the use of [illegible] a hypertonic postfixation rinse and careful dehydration.

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Table I

Experiment II Immersion fixation in 5% glutaraldehyde dissolved in a phosphate buffer containing different tonicities of sucrose and NaCl. The quality of the preservation is rated in terms of »relatively best« (+) and »not useful« ( ) (MIll = Millonig buffer)

Type and tonicity of osmotically active addition / Tonicity of buffer	SUCROSE mOsm						NaCl mOsm					
	0	100	200	300	400	500	0	100	200	300	400	500
100 mOsm MIll	–	–	–	–	–	–	–	–	–	–	–	–
300 mOsm MIll	+	+	+	–	–	–	+	+	+	–	–	–
500 mOsm MIll	–	–	–	–	–	–	–	–	–	–	–	–

Table II

Experiment III Perfusion fixation in 5% glutaraldehyde dissolved in 300 mOsm phosphate buffer containing different tonicities of both sucrose and NaCl. The quality of the preservation is rated in terms of »relatively best« (+) and »not useful« ( )

Tonicity of sucrose (mOsm)	Tonicity of added NaCl (mOsm) 0	100	150	200	300
0			–		–
150	+	–			
200	+		–	–	
250	–				

Table III

Experiment IV Rinsing and osmification. The quality of the preservation as seen after treatment with the different solutions is rated in terms of »relatively best« (+) and »not useful« ( )

	Addition of sucrose (mOsm)				
Rinse	0	200	200	400	400
Osmification	0	0	200	0	400
Rating	–	+	+	–	–

Table IV

Experiment V Dehydration. The quality of the preservation as seen after using different aceton concentrations and dehydration times is rated in terms of »relatively best» (+) and »not useful» ( )

Acetone % 10	20	30	40	50	60	70	80	90	95	100	Specimen	Rating
										120	A	–
								30	0	0	B	–
								60	0	120	C	–
						60	60	60	0	120	D	–
					10	10	10	10	0	30	E	–
					30	30	30	30	0	0	F	–
					30	30	30	30	30	0	G	–
					180	180	180	180	0	720	H	–
				15	0	15	15	15	0	15	I	–
				60	0	60	60	80	60	120	J	–
				30	0	30	30	30	30	120	K	–
		5	0	5	5	5	5	5	5	5	L	–
5	5	5	5	5	5	5	5	5	5	5	M	–
60	60	60	60	60	60	60	60	60	60	120	N	+
					480 (continuous)						O	–

Table V

Mean values and (SD) of the length (mm) of the proximal parts of six S dorsal roots from three cats as found during treatment according to the useful methods

Rinse —— 70	Acetone % 80	90	95	100	After polymerization
15 (± 0)	14,3 (± 0.2)	14,2 (± 0 1)	14 0 (± 0 1)	13 9 (± 0 1)	13,9 (±0,1)

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LEGENDS TO FIGURES

A	astrocyte
BN	borderline node of Ranvier
C	central nervous fibre
CNS	central nervous system
CZ	core zone
EM	electron micrograph
Exp.	experiment
Glut.	glutaraldehyde
Im.	immersion fixation
LM	light micrograph
MIII.	phosphate buffer f Millonig
MZ	mantle zone
P	peripheral nervous fibre
Perf.	perfusion fixation
PNS	peripheral nervous system
SC	spinal cord
Sol.	solution
Sucr.	sucrose
U	unmyelinated axon
V	blood vessel
VC	vehicular composition

Fig. 1 A digest of material, methods and results.

The following pictures show the transitional regions of feline lumbosacral dorsal pinal rootlets. Th fixation agent is 5% glut. unless stated otherwise.

Fig. 2 LM. Toluidine blue stained longitudinal cryostat section of material fixed only with glutaraldehyde. The CNS tissue appears light and protrudes like a con into the darker-stained PNS tissue. The dorsal root-spinal cord junction is in the upper right corner of the picture The hatched line indicates the level corresponding to the cross section of Fig 3 (x130).

Fig. 3 EM. Cross section. Useful methods The CNS compartment occupies the centre of the micrograph. It is characterized by CNS closely spaced nerve fibres forming the core zone (CZ), and peripheral mantle zone (MZ) where there is comparatively large amount of glia cytoplasm. The surrounding PNS compartment is characterized by its more contrast-rich myelin sheaths and more loosely arranged nerve fibres. Blood vessels (V) are restricted to the PNS compartment (750).

Fig 4 EM. Cross section. Perfusion fixation according to the sorginal preparative methods The PNS compartment is seen on the right, the core zone in the middle of the picture Myelinated fibres in the PNS compartment show minor fissures, while myelin beath splitting, axonal retractions (x) and glial defects (*) are common in both the mantle and core zones of the CNS compartment (x1900).

Fig 5 EM. Perfusion fixation according to the soriginal preparative methods Longitudinal section through a large myelinated fibre. Borderline node of Ranvier (BN) in the centre of the picture. The CNS part of the fibre is on the left. The PNS part of the fibre appears well preserved except for a minor empty space in the terminal cuff of the myelin sheath (arrow). The CNS part of the fibre shows multiple myelin fissures. There are large empty-looking spaces (x) outside the nodal region (x4200).

Fig 6. EM. Immersion fixation in the same medium as in Fig 4. Longitudinal section through large myelinated fibre The CNS compartment is on the left. The PNS part of the fibre shows splitting in the myelin sheath at incisures of Schmidt Lanterman and close to the node (arrows), as well as axonal retractions from the inner contour of the myelin sheath (x). The CNS part of the fibre shows extensive myelin sheath splitting and hrinkage defects between the axon and the myelin sheath (*) and empty spaces between the myelin sheath and the surrounding glia cytoplasm (*). Note the difference in preservation quality between the CNS and PNS components (x1700).

The following pictures (Figs 7-33) are all electronmicrographs of cross sections demonstrating the preservation of the transitional region as obtained in some of the preparatory experiments. For details compare with the digest in Fig. 1 The PNS compartment is in the lower part of the pictures (1100).

Fig 7 Exp. 2 Immersion fixation. VC· 100 MilL, 0 sucr 0 NaCl The ultrastructure of the CNS compartment is ruined. CNS myelinated fibres are almost completely destroyed and appear like whorls of myelin lamellae surrounding shrunken axons (arrows). The glial components appear swollen and vacuolated and show disrupted membranes. Note the comparatively good preservation of the PNS fibres (P).

Fig 8 Exp. 2 Immersion fixation. VC: 300 MilL, 0 sucr 0 NaCl CNS fibres show myelin sheath splitting. The surrounding glia cytoplasm reveals some empty spaces. The degree of preservation was classified as being among the »relatively best» ones

Fig 9 Exp 2. Immersion fixation. VC: 500 MilL, 0 sucr 0 NaCl Myelin sheaths are compact but show deformed contours. Several axons are seriously shrunken (x). Torn empty spaces (*) are prevalent outside the CNS fibres.

Fig. 10 Exp. 2 Immersion fixation. VC: 300 MilL, 100 sucr 0 NaCl CNS myelin sheaths show minor fissures. Axonal retractions (x) from the inner contour of the myelin sheath are common. The preservation degree was classified as being among the »relatively best»

Fig 11 Exp. 2 Immersion fixation. VC. 300 MilL, 200 sucr 0 NaCl The CNS fibres show faint fissures in the myelin sheaths. Axonal retractions (x) are uncommon and the glia ppears free of large empty spaces. The PNS fibres are well preserved. The preservation degree was classified as being among the »relatively best»

Fig. 12 Exp. 2 Immersion fixation. VC: 300 MilL, 500 sucr 0 NaCl The CNS fibres show large myelin fissures as well as general myelin sheaths splitting The axons have shrunken to irregular star-shaped dark profiles. Glial cytoplasm has shrunk to a dark reticulum leaving large empty spaces in between the fibres. In contrast to this PNS myelin sheaths appear more or less intact, whereas the axons are seriously shrunken. Compare with Fig. 14.

Fig 13 Exp. 2 Immersion fixation. VC. 300 MilL, 0 sucr 200 NaCl The CNS fibres show compact myelin sheaths. Axonal retractions (x) from the inner contour of the myelin sheath do occur The PNS fibres ppear well preserved. The preservation degree was classified as being among the »relatively best»

Fig. 14 Exp. 2 Immersion fixation. VC: 300 MilL, 0 sucr 500 NaCl. The CNS fibres show compact myelin sheaths and axonal retractions are comparatively rare. The PNS fibres ppear well preserved Compare with Fig 12.

Fig 15 Exp 3 Perfusion fixation. VC 300 MilL, 0 sucr 150 NaCl The CNS fibres show large myelin fissures. Torn empty spaces are seen in the glia outside the myelin sheath (x). PNS myelinated fibres appear well preserved.

Fig 16 Exp. 3. Perfusi n fixation VC. 300 MilL, 250 sucr 0 NaCl In the CNS compartment there are many large myelin fissures. Torn empty spaces are rare in the glia. PNS myelinated fibres are well preserved

Fig. 17 Exp. 3. Perfusion fixation. VC. 300 MilL, 200 sucr 200 NaCl In the CNS compartment there is a generalized myelin sheath splitting. The axons have mostly retracted from the inside of the myelin sheaths (x) and the glia contains some large empty spaces (*). PNS myelinated fibres appear well preserved.

Fig 18 Exp. 3. Perfusion fixation VC. 300 MilL, 200 sucr 0 NaCl In the core zone (CZ) the fibres ppear with few and small fissures in the myelin sheaths and the glia appears more or less free of empty spaces. Glial defects (x) and myelin sheath splitting are seen in the mantle zone (MZ)

In the PNS compartment the fibres appear well preserved. The preservation degree was classified as being the »relatively best» one

Fig 19 Exp. 4 Rinsing after glut fixation. The rinsing solution was a 300 MilL buffer to which 400 sucr had been added CNS myelinated fibres show a pronounced and generalized myelin sheath splitting (cf. Figs. 12, 18 and 20). The PNS compartment appears well preserved

Fig 20 Exp 4 Rinsing after glut. fixation. The rinsing solution was a 300 MilL buffer to which 200 sucr had been added. In both the core zone (CZ) and the mantle zone (MZ) the myelin sheaths show only small fissures. Empty spaces have to be searched for. PNS fibres are well preserved The preservation degree was classified as being the »relatively best» one

Fig 21 Exp. 5. Dehydration. E in Table IV Short time in few acetone steps. In the CNS compartment most fibres show splitting in the myelin sheath. Axonal retractions from the inner contour of the myelin sheath (x) as well as empty spaces outside the myelin sheath (*) are seen. The PNS compartment appears well preserved.

Fig 22 Exp 5. Dehydration. M in Table IV Short time in many acetone steps. CNS fibres show large multiple myelin fissures. The fibres in the PNS compartment are well preserved.

Fig. 23 Exp. 5. Dehydration. H in Table IV Long time in few acetone steps. In the CNS compartment splitting is seen t sites in the myelin sheaths. The PNS compartment is well preserved.

Fig 24 Exp 5. Dehydration. 0 in Table IV Eight hours continuous dehydration. CNS fibres show large fissures. Axonal retractions from the inner contour of the myelin sheath (x) and defects in the surrounding glia cytoplasm (*) are prevalent. In the PNS compartment the fibres show fissures in some myelin sheaths.

Fig 25 Exp. 5. Dehydration. F in Table IV Dehydration stopped in 90% acetone and embedding started here. In the CNS compartment the fibres appear collapsed with a thin dark condensed rim of cytoplasm surrounded by a well-preserved myelin sheath of low electron density In the PNS compartment the fibres appear less collapsed. The myelin sheaths are well preserved and more electron-dense. The axoplasm is clear

Fig. 26 Exp. 5. Dehydration. N in Table IV Long time in many acetone steps. The CNS compartment appears to be almost free of axonal retractions, of myelin sheath splitting and of tissue defects in the adjoining glia. The largest myelin sheaths, although quite compact, show a somewhat serrated outline. PNS fibres revealed occasional splittings at the incisures of Schmidt Lanterman. The preservation degree was classified as being the relatively best one. This is also the procedure which finally was classified as the useful methods.

Fig 27 Exp. 6. Embedding. Araldite. CNS fibres show axonal retractions (x) and generalized splitting in the myelin sheaths. Empty spaces are prevalent in the glia (*). PNS fibres are well preserved.

Fig 28 Exp. 6. Embedding Spurr CNS myelin is of a comparatively low contrast. CNS myelin sheaths show generalized and severe splitting. PNS fibres are well preserved.

Fig 29a Additional exp. Addition of 0.2% digitonin to the glut. post fixation and rinsing solutions in a preparative procedure otherwise analogous to that used in Fig. 26. The plasma membranes of the Schwann cell, axon and astrocyte (A) are disrupted and the cytoplasmic compartments contain vesicle-like entities (x) (x27100).

Fig 29b Specimen treated according to the same preparative procedure as in Fig. 26 (x27100).

Fig. 30 Additional experiment. 1% formaldehyde and 4% glut. used as fixation agent. CNS axons have retracted from the inner contour of the myelin sheath (x). Myelin sheath splitting and glial defects (*) are prevalent The PNS fibres ppear less distorted (cf. Figs. 9 and 14).

Fig 31 Additional experiment. Dehydration in ethanol. CNS fibres show a generalized and pronounced myelin sheath splitting Axonal retractions (x) and glial defects (*) are prevalent. PNS fibres are well preserved.

Fig 32 Additional experiment. Embedding in GMA. CNS fibres show extensive myelin sheath splitting The glia is deformed and shrunken and contains large empty spaces (x). The PNS fibres are well preserved.

Fig. 33 Additional experiment Embedding in resorcinol formaldehyd (Reform I) Stained *en bloc* in uranyl acetate and sectioned stained in lead citrate The specimen revealed a negative contrast pattern with low-density myelin sheaths and high-contrast glia and axoplasm

Figures at the end of this volume

Running title: EM preparation of the transitional region

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From the Department of Anatomy Karolinska Institutet, Stockholm Sweden

Observations on the Morphology at the Transition between the Peripheral and the Central Nervous System in the Cat. II

General organization of the transitional region in S dorsal rootlets.

by

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ABSTRACT

C H. BERTHOLD and T CARLSTEDT *Observations on the morphology at the transition between the peripheral and the central nervous system in the cat. II. General organization of the transitional region in S dorsal rootlets.* Acta physiol. scand. *1977 Suppl. 446* 23-42.

The proximal segment of feline S_1 dorsal rootlets were investigated light and electronmicroscopically A cone shaped protrusion of CNS tissue was found to extend distally into each rootlet for distance of 100-1000 µm. That free segment of a rootlet which when cross sectioned revealed both CNS and PNS constituents was denoted the transitional region (TR). The TR was subdivided into an axial CNS compartment and a surrounding PNS compartment. Th CNS compartment consisted of a core zone that showed the organization of CNS fiber tract, and of an outer astrocytic mantle zone which corresponded to the external glial limiting membrane of the brain and the spinal cord. The outer aspect of the mantle zone constituted the PNS-CNS borderline. The mantle zone was rich in astrocytes and projected large number of thin processes — the glial fringe — distally into the endoneural space f the PNS compartment. There were large fenestration defects in the basement membrane that demarcated the endoneural space from the glial pace. The mantle zone contained many large myelinoid bodies and som encapsulated small dark unclassified cells Similar cells, some covered by a basement membrane, were t hand in the adjacent endoneural space The myelinated nerve fibres were equipped with node of Ranvier at their borderline passage. During their course proximally in the TR the unmyelinated axons were redistributed from random arrangement to a more or less segregated position superficially in the ventrolateral aspect of the rootlet. Crossing the borderline ment a considerable increase in the umber of small myelin ted nerve fibres on the CNS side and a corresponding decrease in the number of unmyelinated axons.

The occurrence of CNS tissue in the proximal parts of spinal roots and cranial nerves has been documented in a large number of lightmicroscopical investigations (see for instance Virchow 1858, Obersteiner and Redlich 1894 Hulles, 1906; Skinner 1931, Tarlov 1937 a, b). According to the reports on higher vertebrates and man the CNS tissue protrudes more peripherally in sensory than in motor nerves the eight cranial nerve and the dorsal spinal root S_1 being equipped with particularly extensive CNS parts (Skinner 1931 Tarlov 1937 b). The contour of the PNS-CNS borderline is described as »helmet like» (Tarlov 1937 b) or »dome-shaped» (Skinner 1931), its convexity facing the PNS. The tissue zone forming the curved borderline has been reported to constitute a »double *lamina cribrosa*» (Tarlov 1937 a), which outer part derives from the *pia mater* and covers an inner glial layer Solitary »islands» of glial tissue are reported to occur in the endoneural space close to the PNS-CNS borderline. (Tarlov 1937 a, see also

Maxwell *et al.* 1969). The cell content of the transitional region is stated to be comparatively high (Tarlov 1937 a) and oligodendrocytes have been suggested as the dominant glial element (Tarlov 1937 a) The myelinated nerve fibres were found to be constricted as they pierced the »*lamina cribrosa*» and were claimed to lose their myelin for the first 10-60 μm of their CNS part, this giving rise to the so-called »Aufhellungszone» (Hulles, 1906) The first indication that the myelinated fibres were equipped with a node of Ranvier at their passage across the PNS-CNS borderline was given by Friede (1961)

Electron-microscopic works on this region of the nervous system are for reasons which probably have to do with difficulties in obtaining good ultrastructural preservation quite sparse (Foncin 1961, Nataniel and Nataniel, 1963, Maxwell *et al* 1969- Ross and Burkel, 1971 Steer 1971). In the first EM study on the transitional region Foncin (1961 — rat) demonstrated the PNS-CNS borderline nodes and clearly showed that the »Aufhellungszone» was an artifact based on pronounced myelin sheath splitting on the CNS side (see also discussion in Carlstedt, 1977). More recent EM reports (Maxwell 1967 Maxwell *et al.*, 1969-monkey- Ross and Brukel, 1971-rat; Steer 1971-rat) have shown that the *pia mater* does not participate in the formation of the »*lamina cribrosa*» Contrary to the light-microscopical opinions, oligodendrocytes were rarely observed, the dominating glial cell being the astrocyte which formed a feltwork of interwoven processes in the border layer corresponding to the external glial limiting membrane of the brain and the spinal cord.

It can be concluded that the ultrastructural organization of the very region where PNS tissue meets CNS tissue is far from fully explored and has not been investigated in the cat. This region holds points of great interest with regard both to neuroanatomy and to neuropathology and offers the unique opportunity to analysing one and the same axon as it appears in the peripheral as well as in the central nervous system For an up to date review and discussion regarding the transitional region see Gamble (1976).

The organization of the transitional region seems furthermore to be of importance in *tabes dorsalis* (Obersteiner and Redlich, 1894) in primary myelin degeneration (Prineas *et al.* 1969) and in tumour development (Mincler 1972). A detailed knowledge of the ultrastructure of this region is a *sine qua non* when discussing and investigating the highly interesting issue of a possible immigration of Schwann cells into the CNS (see, for instance Gilmore 1971 Ghatak *et al* 1973, Hori 1973, Wood *et al.* 1975- Blakemore and Patterson 1975; Raine 1976).

The present work which makes use of a recently developed preparatory procedure (Carlstedt, 1977) deals with the general ultrastructural organization of the transitional region (TR) and investigates the distribution of cell units and nerve fibers in the TR.

MATERIAL AND METHODS

Eight adult cats were anaesthetized by intraperitoneal injection of pentobarbital 40 mg/kg BW and artifically ventilated with 100% oxygen. The lumbosacral spinal cord and its roots were fixed by vascular perfusion through the thoracic aorta at controlled intermittent pressure (Berthold, 1968). The fixative agent was 5% glutaraldehyde dissolved in a 300 mOsm Millonig buffer with the addition of 2 7% low-molecular Dextran and 200 mOsm sucrose After laminectomy the specimens consisting of the first sacral spinal cord segment and its dorsal roots were removed.

Specimens intended for cryostat sectioning (2 cats) were, after a short rinse in 300 mOsm Millonig buffer, frozen and longitudinal sections 15-20 μm thick were cut in a cryostat t -30°C. The sections were placed on glass slides and stained in alkaline toluidine blue (Carlstedt, 1977).

Specimens intended for Vestopal embedding (6 cats) were trimmed with a razor blade so the proximal part of each rootlet, bout 15 mm, and its attachment to the spinal cord with a piece of the white matter were left for investigation. After a postfixation for 4 hours in an ice-cold solution of the same composition as used for the perfusion, the specimens were rinsed overnight, treated in a 2% OsO phosphate buffer solution, dehydrated in a slow and stepwise way in acetone and finally embedded in Vestopal (for details see Carlstedt, 1977). The specimens were trimmed into mesas 30 μm high using LKB 11800 Pyramitome and sectioned with a glass knife on a LKB 4800 Ultrotome. The specimens were carefully orientated for cross-sectioning: the nerve fibres were cut perpendicularly to their longitudinal axis and then sectioned in the distal to proximal direction. Longitudinal sections parallel to the longitudinal axis of the nerve fibres were obtained by sectioning the rootlet in the dorso-ventral direction.

Sections for light microscopy (0.5 μm thick) were placed on glass slides and stained with alkaline toluidine blue and examined and photographed in a Leitz photomicroscope.

Besides several series of 25-100 consecutive ultra-thin cross and longitudinal sections, four long series of cross sections, comprising 10,000 (Rootlet 2, cat K1039) 6,000 (Rootlet 3, cat K1040) and 4,500 (Rootlet 1 cat K1028) sections respectively and four long series of longitudinal sections of 200-1000 consecutive sections each were cut. The section thickness was obtained from the ratio between mesa hight and the number of sections it yielded and was found to vary between 600 and 800 Å. The loss of sections was less than 1%.

Th specimens used for the cross section series consisted each of rootlet comprising 100-400 myelinated fibres as determined before serial sectioning in the light microscope.

The sections were placed on one-hole copper grids coated with carbon-stabilized formvar film. After a double staining with uranyl acetate (Brody 1959) and lead citrate (Reynolds, 1963) the sections were examined in a Philips EM 300 electron microscope operated at 80 kV and equipped with a 30 μm objective aperture.

Definition of terms and quantitative estimations

The terms proximal and distal will be used with respect to the spinal root — spinal cord junction (Fig. 1).

Four main cross-sectional reference levels — A, B, C and D (see Fig 1) — will be used with respect to position in the proximal part of the investigated rootlets. Thes

A is that level where the rootlet meets the most caudal part of its corresponding spinal cord segment

B is level situated roughly halfway between A och C.

C is the level at which the glial fringe is situated and which separates the stereotype PNS part of a rootlet from its transitional region and

D is a level some 200-400 μm distally to level C i.e. situated well in the stereotype PNS part of the rootlet.

The additional level 0 is the level at which th tip of the CNS compartment is situated.

Each cross-sectional level — as it appeared in electronmicrographs at X 2000 magnification — was divided, with respect to its centre, into dorsal and a ventral part and each of these was divided in medial and lateral part. So each level was separated into four parts or quasi-quadrants (see Figs. 21a, 22a, 23a). In view of the unsymmetrical shape of the cross sections and the differences in shape between the various levels, true quadrants of comparabl size could not be obtained. Hence, in order to make a comparison of the fibre distributions in different parts of a cross-sectional level and at different levels of the transitional region at all possible the area of each separate quasi-quadrant was estimated by planimetry and the relative occurrence of nerve fibres per unit area calculated (see legend to Fig 24).

In order to evaluate th distribution of different fibre classes with respect to depth in the rootlet, each cross-section level was divided into four concentric areas: one central spot surrounded by three concentric zones of equal breadth and assimilated to the contour rootlet (see F gs. 21f, 22f, 23f). For the same reason as given above, the areas spot and the surrounding zones were determined by planimetry

General organizatio of the trans l region

The nerve fibres were separated into *myelinated and unmyelinated (C-) fibres. The former* group was further separated into large (> 13 µm thick), medium (6-13 µm thick) and small (< 6 µm thick) fibres. The number and types of the fibres were determined on photocopies representing 20 µm-intervals cross sections through the transitional region (see Figs. 21d 22d, 23d). In addition to this the distribution of the fibres with respect to quasi-quadrants and concentric zones was estimated in the four main cross-section levels A-D (see Figs. 21 23, e, f).

Schwann cells and fibroblasts were identified according to the criteri given by Thomas (1963) Glial cells were classified according to the principles of Mugnaini and Walberg (1961) and Peters *et al.*, (1970). The number of the different cell types was estimated from the occurrence of cell nuclei at consecutive 20 µm intervals along the transitional region, using photocopies of cross sections. The occurrence of astrocytes nd oligodendrocytes was moreover checked every 2.5 µm directly in the electron microscope Hence it was possible to estimate the total number of these two cell types in the transitional region, as their cell nuclei are longer than 2.5 µm (cf. Cammermeyer 1960).

RESULTS

General description of the transitional region and presentation of a terminology

The dorsal root S_1 ran more or less parallel with the spinal cord from its ganglion up to the lower level of the segment S At a distance of 5-10 mm from the spinal cord junction the root split into a fan of from five to ten differently sized rootlets which turned medially and lined up in the dorsolateral sulcus (Figs. 1 2). The angle between the length axis of the rootlets and the longitudinal axis of the spinal cord measured about 30°

At a distance of 1 2 mm from the cord the larger rootlets split into 2-3 thinner fascicles, here denoted mini rootlets. These mini rootlets reunited again at the spinal cord junction (Fig 3). Toluidine blue staining of frozen sections gave the peripheral myelin sheaths a dark blue colour that contrasted sharply against the pale blue staining of the central myelin (Fig 3) (cf. Feigin and Cravioto 1961). This difference in the staining properties of the myelin greatly simplified the identification of the central nervous tissue, which extended for some 100-1000 µm from the spinal cord out into the rootlets (Fig 3) In larger rootlets the CNS tissue showed a more rounded end than it did in thinner rootlets, forming a top angle of some 40° and some 20° respectively The thinner the rootlets the shorter was the extension of CNS tissue.

The S dorsal rootlets consisted of CNS tissue at the level of their spinal cord junction (see Fig 1) In most cases the wedge shaped most proximal piece of a rootlet that connected it to the spinal cord (Fig 1) did as well consist of PNS tissue At a cross level about 100-1000 µm more *distally (level C Fig. 1) the rootlet* appeared completely free of CNS constituents. That segment of the free proximal end of a rootlet which contained both CNS and PNS constituents (i.e. the region found in between the reference levels A and C of Fig 1) was referred to as the transitional region. TR (Figs. 4 5). According to this definition the connecting piece is outside the transitional region.

The transitional region (TR) consisted of two main parts: (1) A cone shaped CNS compartment which tapered to a tip (level 0 of Fig 1) in the distal direction and which lacked an endoneural space. (2) A PNS compartment which covered and surrounded the CNS compartment. The PNS compartment contained an endoneural space and a fringe of glial processes which emanated from the CNS *compartment,* and tapered to a tip in the *distal direction (reference level C of* Fig 1)

The interface between PNS compartments was denoted the PNS-CNS borderline. (Figs. 12, 13).

The CNS compartment of the TR disclosed two zones again concentrically arranged. (1) an inner core zone (Figs. 5 9) composed of closely packed central nerve fibres and a few scattered glial cells thus showing the arrangements of a central fibre tract and (2) an outer covering mantle zone 20-30 μm thick and built up of astrocytes and nerve fibres (Figs. 5 9 10). The astrocytes of the mantle zone gave off numerous filiform cytoplasmic processes the glial fringe (Figs. 1 5) which ran distally in the endoneural space parallel to the nerve fibres of the PNS compartment.

The myelinated nerve fibres, adopted a more or less typical paranodal arrangement as they approached the CNS compartment. Just outside the mantle zone the peripheral myelin sheaths ceased and each fibre showed a node of Ranvier at the site where it crossed the PNS-CNS borderline: the borderline node of Ranvier (Figs. 5 8) The axons became again invested by myelin and penetrated the mantle zone in order to reach the central core and eventually the spinal cord. At the passage through the mantle zone the myelinated nerve fibres were loosely packed with comparatively numerous astrocytic perikarya situated in between them (Figs. 5 9 10)

Electronmicroscopic examination

PNS compartment

The PNS compartment was separated from the subarachnoid space by the root sheath. This, the outermost layer of the TR consisted of loosely arranged flattened cells and collagen fibres. No obvious demarcation was noted between the root sheath and the *pia mater* at the root-cord junction (Fig. 5). The endoneural space continued uninterrupted into the subpial space (cf. Heller *et al.*, 1972). The endoneural space of the PNS compartment was comparatively roomy and contained astrocytic processes. the glial fringe (Figs. 5 6, 7) The blood vessels of the endoneural space did not follow the course of the nerve fibres into the CNS compartment (see Table I) Instead the vessels deviated out from their submerged positions and joined vessels situated on the spinal cord surface at the attachments of the rootlets. The CNS compartment of the TR was always found completely free of vessels (Table I, Figs. 6-11).

The passage from the stereotype root region into the PNS compartment of the TR was signalled by the appearance of the cross-cut tip of the glial fringe (Fig 7). The tip was formed by one single or just a few closely spaced cross-cut polygonal astrocytic processes which were demarcated from the endoneural space by a basement membrane. It was in all cases possible by making use of consecutive sections to ascertain that these seemingly isolated glial islands in fact originated from the mantle zone (see below).

More proximally in the TR the glial fringe occupied an increasingly large area of the cross section and its organization became more complex (Fig 8). The number of astrocytic processes increased and the processes joined together forming fenestrated walls in the endoneural space. At a distance of 5-20 μm from the PNS-CNS borderline the glial walls united outside the fibres, separating them and enclosing them together with a minute concentric endoneural space in a tube- or holster like fashion (Figs. 5 8, 9 10 12) In this way the paranodes of most myelinated fibres were found in a narrow endoneural *cul de sac* that extended up to the PNS-CNS borderline and which at its proximal blind end contained the borderline node of Ranvier (Fig. 5).

Extensive glial processes and comparatively deep endoneural *cul de sacs* characterized the glial fringe as far proximally as level B (Fig. 12). From this level onwards the length of the processes decreased and the endoneural *cul de sacs* became shallow measuring only a few μm in length. Hence proximally to level B, where the majority of the smaller fibres entered the CNS compartment, the CNS-PNS borderline was as a rule comparatively easy to observe (Fig 13)

The astrocytic processes forming the glial fringe contained large bundles of glial fibrils clusters of tubules scattered glycogenlike particles, small mitochondria and numerous gliosomes some of which had prismatic cristae (Fig. 16) (cf Morales and Duncan 1971) In the case of one very thin rootlet analysed quantitatively (rootlet 1 Fig 21b) four astrocytic perikarya were noted in the glial fringe. Otherwise the finge was free of astrocytic perikarya.

The glial processes lay closely packed inside a basement membrane and were separated by a narrow empty-looking space 100-200 Å in width Desmosomes and gap junctions (see Nabahima *et al.*, 1975) were common (Figs. 15 18) At some sites however there were larger spaces in a group of glial processes. Several such spaces contained longitudinally-orientated bundles of collagen fibres (Fig 15) As found in series of consecutive sections the collagen bundles originated in the endoneural space and projected several μm into the glial space At these sites the basement membrane that separated the endoneural space from the glial space was fenestrated, thus letting the collagen fibres through Similar basement membrane defects but not associated with traversing collagen bundles, were rather common in the fringe close to the mantle zone (Fig 18). Most collagen fibres measured 200-300 Å in diameter At some sites and particularly in a zone adjacent to the CNS compartment the collagen fibres varied from 200-12000 Å diameter (Fig 15).

The following familiar cell types were constantly noted as members of the PNS compartment: Schwann cells, fibroblasts and pericytes (Figs. 21a 22a, 23a). To this was added the likewise constant occurrence of a cell type of unknown origin and of unknown classification. These unclassified cells were found in the glial fringe and superficially in the mantle zone (Fig 19) They were dark, round, about 5 μm in size and contained a comparatively large, often deeply indented nucleus whose abundant chromatin was distributed in irregular clumps inside the nuclear membrane. The dark, finely granular cytoplasm formed a thin rim outside the nucleus It contained few organelles and formed a few short processes Several of these cells were equipped with a more or less intact basement membrane. In spite of careful serial section analysis it was not possible to demonstrate any contacts between unclassified cells and axons

CNS compartment

Mantle zone. The mantle zone constituted the outer part of the CNS compartment (Fig 5) and measured 20-30 μm in width. It contained the first CNS segments of passing nerve fibres, 95-100% of all astrocytic perikarya of the TR, a large number of myelinoid bodies a few cells of the unclassified type referred to above and a dense outer feltwork of astrocytic processes which without clear delineation, projected distally into the endoneural space forming the glial fringe (Fig 12) Desmosomes and gap junctions were prevalent.

At those rare sites where the plasma membrane of an astrocytic perikaryon directly faced the endoneural space (Fig 13) it was covered by an incomplete basement membrane reflected on to it from penetrating nerve fibres and again lifted away by emerging fringe processes Numerous electron-dense hemidesmosome-like specializations at the astrocytic plasma membrane were present subjacent to the basement membrane (for discussion regarding these entities see Bondareff and McLone, 1973)

The astrocytes were of the fibrillar type (Figs. 12 13) Their perikarya measured 7 12 μm and contained a pale irregularly shaped nucleus, a well-developed Golgi complex, single strands of granular and smooth endoplasmatic reticulum, some small mitochondria (0.2 1 μm in size) fibrils, microtubules electron-dense bodies 0.1 1 μm in size, of which some elongated ones revealed an interior of longitudinally orientated lamellae and numerous gliosomes 1 10 μm in size (cf. Mugnaini and Walberg 1964 Peters *et al.*, 1970) (Figs. 12, 13, 16 17)

There were many myelinoid bodies in the mantle zone (Fig 14) These bodies were irregularly shaped, measured 2 10 μm in size, and revealed a lamellated texture. Intact regions showed an interlamellar period of 100 Å. Most bodies disclosed some intralamellar splitting and crescent-like empty defects at their outer aspects. Some bodies were compact. Others showed a clear centre. The bodies were surrounded by astrocytes.

Core zone. In the core zone, unmyelinated and myelinated fibres were densely packed with scanty of glial cytoplasm in between them (Figs. 6, 9 10 11, 20). Adjacent myelin sheaths sometimes formed an intraperiod dense line along their contact lines. Most myelinated fibres were separated by thin flat astrocytic processes containing bundles of filaments and gliosomes Oligodendrocyes of an ultrastructure fully in line with the current picture (see Mugnaini and Walberg 1964, Peters *et al.*, 1970 and legend to Fig 20) were the dominant celltype. Other cells classified as microglia were occasionally found in the proximal parts of the core zone (Figs. 21a-23a) A few fibrillar astrocytes were also noted in the region close to or proximally to level A (Figs 21b-23b)

Quantitative analysis

One small rootlet (rootlet 1) and two medium sized rootlets (rootlets 2, 3) were investigated quantitatively with regard to the occurrence of different cell types at different TR levels and to the size, shape and extent of the various TR compartments, as well as to the distribution of unmyelinated and myelinated fibres at different TR levels (Figs. 21 24).

The distance between the reference levels A and C — i.e. the length of the transitional region — was 340 460 and 260 μm respectively (Figs. 21 22, 23). The free length of the glial fringe — i.e. the distance between level C and the tip of the CNS compartment — was 140 40 and 20 μm respectively (Figs. 21d-23d).

In all three rootlets there extended a thin lateral crescent-shaped compartment of PNS tissue proximally to level A. In rootlets 1 and 2 the PNS crescent extended proximally for about 40 μm. It amounted to less than 5% of the total cross section area at level A. More than 95% of the myelinated fibres and most of the unmyelinated fibres has crossed the PNS-CNS borderline distally to level A (Figs. 21d, 22d).

In rootlet 3 the PNS crescent extended proximally for 120 μm. At level A it made up about 20% of the cross sectional area and contained some 20% myelinated fibres and a large number of the unmyelinated axons (Fig. 23d)

Mantle area of the CNS compartment

The CNS compartments of rootlets 1 and 3 were cone-shaped (cf. Figs. 21d, 23d). The size of the mantle area of this cone can be obtained approximately by calculating the mantle area of the cone the height of which is the length of the CNS compartment and the basis radius is obtained from the length of the circumference of the CNS compartment in level A assuming a circular cross section area (Table II). By this method the top angles of the two CNS compartments were estimated to be about 20° and 40° The CNS mantle area was approximately 2.2×10^4 and 7.7×10^4 μm in rootlet 1 and rootlet 3 respectively

In rootlet 2, where the CNS compartment extended distally for 420 μm, the CNS cross section area at level A and at a level 180 μm more distally were about the same. Hence as shown in Fig 22d, the shape of this CNS compartment is roughly that of a proximal cylinder 180 μm in length which tapers off distally into a cone, 240 μm in length the top angle of which can be calculated at about 40° and the mantle area of which is about $7.7\ 10^4$ μm² the total CNS mantle area being 18×10^4 μm (Table II).

General organization of the transitional region

Cell population

The cell population of the transitional region was dominated by Schwann cells (Figs. 21 a, b c 23a, b c) Astrocytes were with very few exceptions present only in the mantle zone The number of astrocytes was 27 103 and 55 in rootlet, 1 2 and 3 respectively Oligodendrocytes were restricted to the core zone The number of oligodendrocytes was 6, 48 and 25 in rootlet, 1 2 and 3 respectively Microglia and pericytes were few the former restricted to the CNS compartment and the latter to the PNS compartment. Unclassified cells lodged in the fringe and in the outer part of the mantle zone They were found at all levels The highest occurence of unclassified cells in one cross section was noted distally in the TR of rootlet 2, where seven individuals were found outside a CNS compartment whose cross section measured 4000 μm^2 (Fig 22a)

Nerve fibres

The rootlets contained 441 1300 and 1359 axons, of which 104 350 and 349 respectively were myelinated at the level C Thus about 25% of all fibres were myelinated. Of these roughly half the number were small and half the number were medium sized. Fibres 13 μm or more in diameter i.e. large fibres, were rare and amounted only to a few per cent (Table I)

The number of axons at level A equalled that of level D or C. However at level A the number of unmyelinated fibres showed a decrease of about 5% as compared to level C The myelinated fibres on the other hand showed a corresponding increase. Figs. 21e, f 23e f and Table I show that this increase concerned only the group of small myelinated fibres which increased by 13 (25%) 46 (27%) and 41 (24%) members in rootlets 1 2 and 3 respectively

The countings of the nerve fibres at 20 μm intervals along the transitional region showed that the larger fibres entered the CNS distally to the thinner ones (Figs. 21d-24d) For example in rootlet 2 30% of the medium-sized and 55% of the small myelinated fibres together with some 90% of the C-fibres remained in the PNS compartment at the level where all (100%) large myelinated fibres appeared on the CNS side of the borderline

The occurrence at different TR levels of the four investigated fibre classes — large, medium and small myelinated fibres and unmyelinated fibres — with regard both to quasi-quadrants and concentric zones i given in Figs. 21e f - 23e, f The large myelinated fibres showed a slight preponderance in the dorsal part of the rootlets both at level D and level A. At the latter level the large fibres seemed to aggregate in the outer zones of the dorso-medial part of the rootlets. Medium-sized fibres were somewhat more frequent in the inner zones of the rootlets. Small myelinated fibres showed at level D an even distribution both with respect to quadrants and with respect to concentric zones. At level A small myelinated fibres seemed to have concentrated in the outer zones of the lateral and, particularly of the ventrolateral aspects of the rootlets.

The unmyelinated fibres disclosed at a distal reference level either as in rootlet 3, a more or less random distribution or as in rootlets 1 and 2, a moderate concentration in the lateral half of the cross section. During the passage in the proximal direction to level A there was observed a clear-cut redistribution of the C-fibres. These axons more or less completely deserted the inner zones of the rootlets and became concentrated in the outer zone with a clear preponderance for its ventrolateral compartment. This is illustrated in Fig.24 which gives the relative C-fibre occurences at different sites in proximal and distal levels as calculated per unit area (1000 μm) (See legend to Fig 24) In rootlet 1 for example, the relative occurrence of C-fibres in the ventrolateral part of the cross section is 157 189 148 and 120 as estimated for the central spot to the outermost concentric zone, of the level C respectively At the level A of the same rootlet the corresponding values are *0 40 150 and 355 respectively Thus the C-fibre occurrence appears to have* decreased to zero in the central zone, decreased by 80% in the adjacent zone and increased about three times in the outer zone

DISCUSSION

Terminology

The place in a spinal root or a cranial nerve where the CNS meets the PNS carries many names: the *Aufhellungszone* (Obersteiner and Redlich 1894); the transition zone (Tarlov 1937 Maxwell *et al* 1969) the entrance zone of Obersteiner and Redlich (Bossy 1967) the glioschwannian junction (Mincler 1977) and the glia—Schwann cell border (McFarland and Friede 1971). The present work uses with its own definitions the designation »transitional region» thereby referring to that most proximal free part of a rootlet which in one and the same cross section contains both CNS and PNS tissue This designation has been adopted for the following reasons. »Region» was preferred to such terms as zone junction and border because it was felt to be associated with a more extended and spacious structure than is indicated by the other terms »Transitional» was considered to give greater emphasis to the conceptual importance of the structural changes that take place at the crossing than does, for instance, the word »entrance» The use of designations like »glioschwannian junction» and »glia-Schwann cell border» appeared to be rather awkward, partly because they refer to only one aspect of TR organization and partly because electron microscopy revealed a very complex glia-Schwann cell border» i.e the glial fringe where glial processes to a depth of up to 200 μm interdigitated with the Schwann cells Such terms as »*Aufhellungszone*» and »*lamina cribrosa*» must be considered meaningless and should be avoided. Both are based on preparation artifacts: the *Aufhellungszone*» develops as a result of extensive paranodal CNS myelin sheath splitting and the »*lamina cribrosa*» is the effect of myelin splitting in combination with astrocytic shrinkage (see Carlstedt, 1977)

The concept »transitional region» as here defined includes besides the PNS-CNS borderline a PNS and a CNS compartment. Both these compartments reveal unique morphological features Thus the PNS compartment shows a high cell-body content, profound fibre redistributions and the glial fringe. The CNS compartment is devoid of blood vessels, and carries a mantle zone rich in astrocytes. Moreover crossing the PNS-CNS borderline apparently implies quantitative changes in the number of unmyelinated and myelinated nerve fibres

Blood vessels

The blood vessels of the PNS compartment did not accompany the nerve fibres into the CNS compartment. Nor did this compartment receive vessels from the spinal cord. The PNS vessels deserted the rootlet at its proximal end and joined superficial spinal cord vessels. The reason why the PNS vessels did not enter the CNS compartment is obscure. The observation, however recalls that there are data which indicate that the permeability properties of PNS and CNS vessels are quite different. Thus it has been shown that protein tracers injected intravenously into cats pass easily from the circulation into dorsal root ganglia and spinal nerve roots (Olsson, 1971). In the central nervous system, on the other hand, such extravasation is prevented by the endothelium of the CNS blood vessels ((Reese and Karnovsky 1967 Brightman and Reese, 1969). The lack of blood vessels means that the CNS compartment metabolism has to rely on the diffusion of nutrients and waste products for a distance that may exceed 0.1 mm (cf. rootlet 2)

Unclassified cells and basement membrane

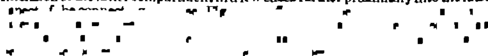
The glial fringe and adjacent parts of the mantle zone contained solitary specimens of a small uncharacteristic cell type which it was found impossible to classify according to current criteria. The observation that several of these cells were invested by a more or less intact basement membrane suggests an epithelial connection and indicates that they may represent some sort of aberrant Schwann cells (cf. Blakemore and Patterson, 1975). However their origin remains for the time being as obscure as their significance.

General organization of the transitional region

The basement membrane which demarcated the astrocytic processes of the glial fringe and the mantle zone form the endoneural space carried large fenestrations. These were at sites several μm in size and formed openings through which endoneural collagen penetrated into the outer layers of the mantle zone. The possible barrier function of the basement membrane at those sites where it constitutes the PNS-CNS interface has been discussed mainly in connection with the occurrence of Schwann cells in the central nervous system (see for instance Gilmore and Duncan, 1969 Blakemore 1975) Obviously the basement membrane of the astrocytes cannot be of any importance as regards diffusion of macromolecular substances. Apart from the fact that it has large fenestrations both ferritin and peroxidase have been found to pass the basement membrane of the external glial limiting membrane (Brightman 1965 Brightman and Reese, 1969) According to Blakemore (1976) the presence of a basement membrane at the PNS-CNS border should prevent migrating Schwann cells from entering the CNS. The present observations suggest that one prerequisite for Schwann cell migration into the CNS — the basement membrane defects — already exists in the apparently healthy cat. Whether the other ingredients — the Schwann cells that are free of axons — are also present in the healthy animal, perhaps in the form of unclassified cells remains to be shown.

Proximal extension of the PNS compartment

In a recent communication Raine (1976) has reported on the occurence in the rabbit lumbosacral spinal cord of rare small islands of PNS tissue characterized by a roomy collagen-containing endoneural space a lack of fibroblasts and a nerve fiber population restricted to unmyelinated and very small (1 2 μm) myelinated axons. These PNS-areas were described as being situated in close proximity to the entry zones of a few dorsal spinal roots. Since serial section analysis was not applied it cannot be decided whether the PNS areas represented true enclosed islands or whether they in fact are but central extensions of the PNS compartment of the TR of adjacent rootlets. The similarity in general organization of the PNS areas as shown by Raine (1976) superficially in the spinal cord of the rabbit, and that presently noted proximally in the PNS compartment of the TR in feline S dorsal rootlets, the extension of the latter compartment in a few cases further proximally into the lateral



The mantle zone

The crossing of the CNS demarcation line means penetration of the external glial limiting membrane This membrane measures 10-30 μm in thickness, consists of a complex feltwork of interwoven fibrillar astrocytes and in the cat contains roughly 0.5-1 astrocytic perikaryon per 10 μm of CNS surface area (Williams, 1975; Carlstedt unpublished observation) In the transitional region, the mantle zone is the counterpart to the external glial limiting membrane

In the present work the size of the mantle-zone area was obtained approximately by calculating the mantle area of the simple geometric figure to which the shape and the size of the CNS compartment could be extrapolated. In rootlets 1 and 3 the configuration of the CNS compartment corresponded roughly to that of a cone whose mantle area was 2.2×10^4 and 7 7 10 μm respectively In line with the astrocytic occurrence (0.5-$1/10^4\,\mu m^2$) elsewhere in the external glial limiting membrane, the mantle zone of the two rootlets should contain 2.2 and 7 7 perikarya respectively Since part of the penetration area is occupied by nerve fibres, it is reasonable to expect still lower values. Actually the mantle zone of rootlet 1 contained 26 astrocytic perikarya and that of rootlet 3 contained 53 i.e the rootlets contained 12 times and 7 times as many astrocytes as expected respectively In the case of rootlet 2 the CNS compartment appeared like a proximal cylinder that was

transformed distally into a cone of the same shape and size as that of rootlet 3. The total mantle-zone area was estimated at $18.7 \times 10^4 \mu m^2$ which gives a maximum of 19 expected astrocytes. Examination of sections at consecutive $2.5 \mu m$ intervals showed that the true value was 95 astrocytes which is about 5 times the expected maximum value. The distribution of the astrocytes in rootlet 2 was, in contrast to rootlets 1 and 3, strikingly irregular: 85 perikarya were present in the mantle of the conical part (length, 240 µm, mantle area $7.7 \times 10^4 \mu m^2$) and 11 in that of the cylindrical part (length, 180 µm, mantle area $11 \cdot 10^4 \mu m^2$). This gives for the conical part an observed value 11 times that expected and for the cylindrical part precisely the expected value of 1.

In all three rootlets, the conical part of the CNS compartment received 85% or more of the myelinated fibres. This is, for instance, illustrated by rootlet 2, where on the other hand only 5-10% of the unmyelinated fibres enter the conical region. Here the majority of the C-fibres penetrates the astrocyte-poor mantle zone of the cylindrical CNS compartment.

Against this background it can be concluded that the mantle zone contains far more astrocytes than expected. This seems in particular to be true of those parts of the CNS compartment which receive the majority of the myelinated fibres. Here the astrocytic occurrence was calculated to be about 10 times the maximal expected value. As indicated by the organization of the cylindrical part of rootlet 2, neither a CNS protrusion as such nor the penetration of hundreds of C-fibres seemed to require an increased number of astrocytes. The high occurrence of mantle-zone astrocytes in the conical part of all three rootlets — there is on average one astrocyte to every 5 myelinated fibres — is difficult to explain. It should, however, be pointed out that myelinated fibres here mean nodes of Ranvier and that the distal parts of the CNS compartment are equipped with an extensive glial fringe of unknown significance.

Distribution of nerve fibres

The large and the medium-sized myelinated dorsal root fibres are known to project mono- or disynaptically to the motoneurons and to join the lemniscus pathways. Some fibres penetrate into the head of the posterior horn of the spinal cord grey matter where they ramify. The small myelinated and the unmyelinated fibres terminate on cells of the extralemniscus pathways or project polysynaptically on motoneurons, particularly on flexor neurons (see Ramon y Cajal, 1909; Szentagothai, 1964; Wall, 1964, Ralston, 1965).

Unmyelinated axons and small myelinated fibres occupy according to Ranson (1913, 1914 a, b) and Ranson and Billingsly (1916) a lateral position in the proximal *part of feline lumbosacral dorsal roots. Earle (1952) reports that unmyelinated feline* dorsal root fibres as they approach the zone of entrance into the spinal cords become separated to the periphery of the root. Wall (1962) on the other hand in a study of feline lumbar dorsal roots doubts that there is any specific localization at all of fibres at the root entrance zone, and Szentagothai (1964) working on the cat and the dog has claimed that the small fibres are grouped both on the lateral and median edges of the rootlets. Sindou *et al.*, (1974) recently noted that, in man, the small myelinated and the unmyelinated fibres were localized in the ventrolateral part of lumbosacral dorsal rootlets.

In the present work the nerve fibres were found to redistribute during their course along the TR with regard to both concentric zone and quadrant position. Since myelinated fibres entered the CNS compartment at more distal levels than did unmyelinated fibres and since the majority of the large and medium-sized myelinated fibres crossed the borderline distally with regard to the majority of small myelinated fibres these, and, in particular unmyelinated axons became aggregated in a thin layer just below the surface of the rootlets between levels B and A. A second shift in position concerned the quadrant distribution of the C-fibres. The calculated values for the relative C-fibre occurrence in the outer zone of the ventrolateral quad-

quadrant at level A was in all cases more than 1.6 times that of the other quadrants (×2.4 1.9 and 1.6 in rootlets 1 2 and 3 respectively) A comparison between distal levels (D in rootlet 2 and 3 C in rootlet 1) and level A shows that the relative C-fibre occurrence in the outer zone of the ventrolateral quasi-quadrant increases some 2-4 times. On the other hand these calculations (see Fig 24 and its legend) shows clearly that comparatively large numbers of C-fibres are present in the outer zones of the other quadrants as well.

Our conclusions disagree with Wall s (1962) opinion that there is no particular segregation of the nerve fibres in the proximal parts of feline spinal rootlets. Nor do the present data lend support to the idea (Sindou *et al.*, 1974) that partial and superficial rhizotomy along the ventral aspects of lumbosacral dorsal roots should be accepted as a neurosurgical procedure to alleviate regional pain. This is a question which cannot be evaluated until the small fibre distribution in human dorsal rootlets has been thoroughly documented by electron microscopy

Fibre number on the two sides of the PNS-CNS borderline

Countings of the number of nerve fibres at the different levels showed that although the total number of fibres was roughly the same at all levels, the number of small myelinated fibres increased by 25% between levels B and A. The increase in myelinated fibres was accompanied by a similar decrease in the unmyelinated fibres This suggests either that some PNS C-fibres acquire myelin presumably as they enter the CNS or that some C-fibres never enter the proximal level of the TR, whereas on the other hand some small myelinated fibres must have branched as they reached the proximal TR level. No straightforward explanation is available at the present time. This would require numerical analysis performed on sections separated by only a few μm and could not, as in the present case, be based on sections separated by up to a hundred or more μm.

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Table I

Number of unmyelinated and myelinated fibres and the occurrence of blood vessels at four cross levels of the free proximal part of S_1 dorsal rootlets in three cats.
(I = large II = medium, III = small fibres)

Specimen	Level	Number of unmyelinated fibres	Number of myelinated fibres Total	I	II	III	Number of vessels
	D	–	–	–	–	–	–
	C	337	104	3	50	51	1
Rootlet 1	B	337	104	3	50	51	0
	A	324	117	3	50	64	0
	D	950	350	10	174	166	6
Rootlet 2	C	950	350	10	174	166	6
	B	950	350	10	174	166	4
	A	904	396	10	174	212	0
	D	1010	349	9	171	169	7
Rootlet 3	C	1010	349	9	171	169	7
	B	1010	349	9	171	169	7
	A	969	390	9	171	210	0

Table II

Estimations of length and mantle area of the CNS compartment and its content of astrocytes

Specimen	Length f CNS compartment (µm)	Appr area of the mantle zone (µm)	Expected number of astrocytes in the mantle zone3)	Estimated number of astrocytes in the mantle zone
Rootlet 1	200	22321	2,2	26
Rootlet 2	420	187205	19	95
		(77406 1)	(7 7 1)	(85 1)
		(109800 2)	(11 2)	(11 2)
Rootlet 3	240	77406	7 7	53

1) Conical part
2) Cylindrical part
3) Based on estimations of feline external glial limiting membr ne (0.9 astrocyte nuclei per 10^4 µm)

General organization f the transitional region

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LEGENDS TO FIGURES

Abbreviations used in figs. and legends

A	Astrocyte
BN	Borderline node of Ranvier
C	Central nervous tissue
Co	Collagen fibre
CZ	Core zone
D	Dorsal
DC	Dorsal column
DH	Dorsal horn
EM	Electron micrograph
GF	Glial fringe
L	Lateral
LM	Light micrograph
M	Medial
MB	Myelinoid body
MR	Mini rootlet
MZ	Mantle zone
O	Oligodendrocyte
P	Peripheral nervous tissue
PN	Paranode
R	Rootlet
RS	Root sheath
SC	Spinal cord
U	Unmyelinated fibres
UC	Unclassified cell

Fig. 1 Schematic drawings howing S_1 dorsal root (I), frontal section through region of S dorsal rootlet (II) nd the distal part of the glial fringe (III).

I. The dorsal root S_1 and part of its spinal cord segment as viewed from beh marked out by the bracket is presented at higher magnification in II.

II. Frontal section through the TR The proximal and the distal direction tudinal axis of the rootlet re indicated. Four reference (A, B, C, D) and one section levels through the proximal part of the S_1 dorsal rootlet are transitional region extends between levels A and C. The dotted line indi rootlet junction (SJ). The wedge shaped part of the rootlet situated bet spinal cord-rootlet junction (SJ) is denoted the connecting piece (Cp). The CNS compartment is situated at level O The tip of the glial fringe is situa runs through the stereotype part of the rootlet some 200-400 μm distal is not drawn to scale.

III. Artists view of glial fringe. Only myelinated fibres are indicat

Fig. 2 Dorsal aspect of the glutaraldehyde fixed lower lumbos adjoining dorsal spinal roots L_6, L_7 and S_1 The left dorsal root square indicates the region shown in Fig 3 (x3).

Fig. 3 LM. Toluidine blue stained longitudinal cryostat section glutaraldehyde fixed S_1 dorsal root. Spinal cord (SC) to the right into several rootlets (R) and minirootlets (MR) before entering the light and protrudes int the darker stained PNS tissue (x17) serrated PNS-CNS borderline (arrows) (x300).

General organization f th transitional regi

Fig 4a LM. Vestopal embedded specimen. Toluidine blue stained cross section of the dorsolateral part of the spinal cord and a number of adjoining S dorsal rootlets. Arrow indicates the dorsomedial sulcus. One rootlet joins the cord in the dorsolateral sulcus (single-bar arrow). The pale central areas of the two medial rootlets are not artifacts but an effect of the different staining properties f CNS and PNS myelin (x110)

Fig 4b Higher magnification of the rootlet as marked out by double-bar arrow in Fig. 4a. The rootlet contains a darker stained outer compartment of PNS tissue (P) and lighter stained inner compartment of CNS tissue (C) (x110).

Fig 5 EM Survey picture of a longitudinally sectioned TR of a thin S dorsal rootlet. The spinal cord is situated in the lower aspect of the picture and the stereotype PNS part of the root in th pper aspect f the picture The five reference levels A, B, O C, D (see Fig. 1) are indicated to the right of the picture The interface between the PNS and CNS compartments of the rootlet is formed by the mantle zone (MZ), which gives off the glial fringe (GF) in distal direction. The glial fringe occupies part of the endoneural space of the PNS compartment. The CNS compartment consists of a core zone (CZ) with closely packed myelinated fibres. Borderline nodes (BN) and CNS borderline paranodes together with astrocytes (A) constitute the mantle zone (MZ) (x1300).

Figs. 6-11 This sequence of electron micrographs shows six different cross levels selected from a complete series of consecutive cross sections through the proximal free part of a S, dorsal rootlet in specimen K 1040 The orientation f the rootlet is given by the reference directions M medial V ventral, I lateral, D dorsal in the compass card given in the upper right corner of each picture The section number (section thickness 700 Å) is given in the lower left corner Seven fibres have been marked out, fibre 2 is a large fibre (> 13 μm), fibres 3 and 5 are medium sized (6-13 μm) and fibres 1 4, 6 and 7 are small myelinated fibres (6 μm) (x1500). Inset shows higher magnification (x6800) of fibre I.

Fig 6 Stereotype PNS part of the rootlet as seen in a cross section 340 μm distally to the proximal upper limit of the TR. This position corresponds roughly to the reference level D as given in Figs. 1 nd 5 There is no clear segregation of various myelinated fibre types. Most of the unmyelinated (U) fibres occur superficially in the rootlet. The rootlet contains 2 vessels (Va).

Fig 7 Distal part f the TR as seen in a cross section 260 μm distally to the proximal limit of the TR This position corresponds roughly to the reference level C (as given in Figs. 1 and 5). The whole cross section is occupied by the PNS compartment. CNS tissue constituents — the tip f th glial fringe — appear l ke several astrocyti processes in the immediate vicinity of bre 1 (see inset) F bre 2 shows paranodal crenation (PN) All fibres are of PNS type

Fig 8 Distal part of the TR as seen in cross section 240 μm distally to th proximal limit of the TR This position corresponds roughly to the reference level O as given in Figs. 1 and 5. There are several deeply crenated PNS borderline paranodes (PN) in between the areas occupied by the glial fringe The borderline node of fibre 2 is found close to the centre of the picture This appeared to be th most distal f all borderline nodes in this rootlet. There is a myelinoid body (MB) localized in the astrocytic compartment to the right f the borderline nod

Fig 9 Distal part of the TR as seen in a cross section 210 μm distally to the proximal limit of the TR This position corresponds to level in between the reference levels O-B. The PNS compartment encloses the CNS compartment. Most large fibres are of CNS type and localized in the centre of th picture The main part f the smaller fibres are in the outer zone of the rootlet and are of PNS type Note the high occurrence f unmyelinated fibres (U) in the most superficial zone f th rootlet. Fibre 1 shows paranodal co figuration (PN) The core zone (CZ) discloses closely packed CNS myelinated fibres. The m ntl zone (MZ) contains borderline nodes (BN) and CNS borderline paranodal segments as well as astrocytes (A). Th glial fringe is situated between the paranodal parts f PNS my linated fibres There are no vessels in the CNS compartment

Fig. 10 The section level is 10 μm more proximal than in Fig. 9 Fibre 1 which already at reference level C was surrounded by astrocytic processes has here som 60 μm further proximally shifted to the CNS type

Fig. 11 Proximal limit of the TR. The position of the section corresponds to the level A of Fig. 1 Arrows point at the two most proximally situated borderline nodes. Th rootlet is free of vessels.

Fig. 12 Cross section through the mantle zone (MZ) in the lower part of the picture and adjacent parts of the PNS compartment. The position of the section is about 20 μm proximally to reference level O (Fig. 1). The PNS-CNS borderline is marked out by the dotted line. A large CNS myelinted fibre and astrocytic perikarya are seen in the mantle zone. The glial fringe (GF) is extensive. Its astrocytic processes forms more or less complete walls between the PNS myelinated axons. Fibres 1 and 2, both cut through their PNS borderline paranodes, are holstered by the glial fringe, each together with a rim of endoneural space that contains collagen fibres (x4000).

Fig. 13 Cross section through the mantle zone (lower half of the picture), and adjacent parts of the PNS compartment. The position of the section is about 20 μm distally to the proximal limit of the TR. The dotted line suggest the course of the PNS-CNS borderline. Two PNS myelinated fibres and collagen (Co) containing endoneural space are enclosed in the thin rim of loosely packed astrocytic processes which forms the glial fringe t this proximal level of the TR (x4700).

Fig. 14 EM. Mantle zone. Two myelinoid bodies (MB) are situated close to the borderline segment of a myelinated fibre (x) (x13000).

Fig. 15. Cross cut bundles of endoneural collagen fibres separated by thin astrocytic septa. The astrocytic plasma membrane lacks its usual basement membrane cover Continuity between the clear space occupied by the collagen and the endoneural space of more distal levels was ascertained by serial section analysis. The size of the individual collagen fibres varies between 200 and 1200 Å. Arrows indicate desmosomes between the astrocytic processes (x22000).

Fig 16 EM. Cross cut glial fringe. The pactures shows two apposed astrocytic processes (Ap Ap_1) The cytoplasm of each process contains many fibrils scattered micro tubules and one gliosome supplied with prismatic cristae (x71000)

Fig 17 EM. Mantle zone. Large gliosome (2 μm) containing a fine granular matrix (x21000).

Fig 18a, b EM. Cross section through the glial fringe showing a large defect in the basement membrane (between arrows) outlining the astrocytic processes The gap between the two pictures is about 5 μm. Gap junti n at the arrow with bar in Fig 18a (x44000)

Fig 19 EM. Cross section through the glial fringe. Unclassified cell (UC) surrounded by basement membrane (arrows) (17000).

Fig. 20 EM. Cross sectioned core zone. In this part of the TR the tissue show the appearence of CNS fibre tract with closely packed nerve fibres and intervening glial elements. An oligodendrocyte (O) classified according to its characteristic appearance (Mugnaini and Walberg, 1964, Peters *et al.*, 1970): a rounded ncleus outline, large amounts of free ribosomes, microtubuli, heterogenous dense bodies and mitochondria but n gliosomes. The cell body contacts the myelin of two adjacent fibres (arrows) (x5000)

Figs. 21 22, 23 Quantitative estimations of various parameters in the proximal part of S_1 dorsal rootlet from each of three cats (Rootlet 1 K 1027 rootlet 2 K 1039, rootlet 3 K1040).

a) Number of fibroblasts, microglia, Schwann cells, unclassified cells and pericytes as found in crossections 20 μm part.

b) Number of astrocytes as found in 20 μm long segments of the transitional region. The numbers in the boxes represent astrocytes found in the core zone. All other astrocytes were parts of the mantle zone

c) Number of oligodendrocytes as found in 20 μm long segments of the transitional region.

d) Occurrence of central myelinated fibres in sections 20 μm apart. The picture also gives the total area of the rootlet (hatched line) and th area of th CNS compartment as found at 20 μm intervals (cf. Fig. 1).

) Distribution of large (I) medium (II) and small (III) myelinated and unmyelinated (C) fibres in the four quasi-quadrants (DM dorsomedial DL dorsolateral, VM = ventromedial, VL ventrolateral) of the reference levels A, B, C, D (see Fig 1). The area of each quadrant is given in arbitrary units. The key value is found in the central part of the picture. Schematic drawings of the cross contour of the rootlet at the diverse reference levels and with the different quasi-quadrants re in the central part of the picture.

f) Distribution of fibres as found in three concentric zones and the central spot of the reference levels A, B, C D (see Fig 1). Same arbitrary area scale as in Fig e. Note that each rootlet has its own area key value.

General organization of the transitional region

Fig. 4a LM. Vestopal embedded specimen. Toluidine blue stained cross section of the dorso-lateral part of the spinal cord and a number of adjoining S_1 dorsal rootlets. Arrow indicates the dorsomedial sulcus. On rootlet joins the cord in the dorsolateral sulcus (single-bar arrow). The pale central reas of the two medial rootlets are not artifacts but an effect of the different staining properties f CNS and PNS myelin (x110).

Fig 4b Higher magnification of the rootlet as marked out by double-bar arrow in Fig. 4a. The rootlet contains a darker stained outer compartment of PNS tissue (P) and lighter stained inner compartment of CNS tissue (C) (x110).

Fig 5 EM Survey picture of a longitudinally sectioned TR f a thin S_1 dorsal rootlet. The spinal cord is situated in the lower aspect of the picture and the stereotype PNS part of the root in the upper aspect of the picture The five reference levels A, B O C, D (see Fig 1) are indicated to the right of the picture. The interface between the PNS and CNS compartments of the rootlet is formed by the mantle zone (MZ), which gives off the glial fri ge (GF) in distal direction. The glial fringe occupies part of the endoneural space of the PNS compartment. The CNS compartment consists of a core zone (CZ) with closely packed myelinated fibres Borderline nodes (BN) and CNS borderline paranodes together with astrocytes (A) constitute the mantle zone (MZ) (x1300).

Figs. 6-11 This sequence of electron micrographs shows six different cross levels selected from a complete series of consecutive cross sections through the proximal free part of a S_1 dorsal rootlet in specimen K 1040. The orientation f the rootlet is given by the reference directions M medial, V ventral, L lateral, D dorsal in the compass card given in the upper right corner of each picture. Th section number (section thickness 700 Å) is given in the lower left corner Seven fibres have been marked out, fibre 2 is a large fibre (13 µm), fibres 3 and 5 are medium sized (6-13 µm) and fibres 1 4 6 and 7 are small myelinated fibres (6 µm) (x1600). Inset shows higher magnification (x6800) f fibre I

Fig 6 Stereotype PNS part of the rootlet as seen in a cross section 340 µm distally to the proximal upper limit of the TR. This position corresponds roughly to the reference level D as given in Figs. 1 and 5 There is no clear segregation of various myelinated fibre types. Most of the unmyelinated (U) fibres occur superficially in the rootlet. The rootlet contains 2 vessels (V)

Fig. 7 Distal part f the TR as seen in cross section 280 µm distally to the proxim l limit of th TR This position corresponds roughly to the reference level C (as given in Figs. 1 and 5) The whole cross section is occupied by the PNS compartment. CNS tissue constituents — the tip of the glial fringe — appear lik several astrocyt processes in the immediate vicinity of fibre 1 (see inset) F bre 2 shows paranodal crenation (PN) All fibres are of PNS type.

Fig 8 Distal part of the TR as seen in cross section 240 µm distally to the proximal limit of the TR. This position corresponds roughly to the reference level O as given in Figs. 1 and 5. There re several deeply crenated PNS borderlin paranodes (PN) l between the areas occupied by the glial fringe The borderlin node of fibre 2 is found close to the centre of the picture This ppeared t be the most distal of all borderline nodes in this rootlet. There is a myelinoid body (MB) localized in the astrocytic compartment to the right f the borderline ode.

Fig 9 Distal part of the TR as seen n a cross section 210 µm distally to the proximal limit of the TR This position corresponds to a level in between the reference levels O-B The PNS compartment encloses the CNS compartment Most large fibres are of CNS type and localized in th centre of th picture The main part of the smaller fibres are in the ter zone of the rootlet and are of PNS type Note the high occurrence of unmy linated fibres (U) in the most superficial zone of th rootlet. F bre 1 shows paranodal configuration (PN) The core zone (CZ) discloses closely packed CNS myelinated fibres The mantle zone (MZ) contains borderline nodes (BN) and CNS borderline paranodal segments as well as astrocytes (A). Th glial fringe is situated between th paranodal parts of PNS myelinated fibres. There are n vessels in the CNS compartment

Fig. 10 The section level is 10 µm more proximal than in Fig 9 F bre 1 whi h already at reference level C was surrounded by astrocytic processes has here, som 60 µm further proximally shifted to the CNS type

Fig. 11 Proximal limit of the TR The position of the section corresponds to the level A of Fig 1. Arrows point at the two most proximally situated borderline nodes The rootlet is free of vessels.

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Observations on the Morphology at the Transition between the Peripheral and the Central Nervous System in the Cat. III.

Myelinated fibres in S dorsal rootlets.

by

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ABSTRACT

C H. BERTHOLD and T CARLSTEDT *Observations on the morphology at the transition between the peripheral and the central nervous system in the cat. III Myelinated fibres in S dorsal rootlets* Acta physiol scand. 1977 Suppl. 446, 43-60.

Myelinated nerve fibres were analysed at their passage of the PNS-CNS borderline in the transitional region (TR) f felin S_1 dorsal rootlets by means of light and electron microscopy At the borderline, PNS internodal length was 30% shorter than noted further distally in the root. Analysis of variance based on the numerical estimation of various fibre parameters did not support the current idea that a myelinated nerve fibre decreases in diameter as it passes from the PNS into the CNS. There was a rough rectilinear correlation between axon size and number of myelin lamellae in small and medium sized fibres. In large fibres the number of myelin lamellae was highly variable and could not be predicted from axon size. The nodes of Ranvier found at the borderline showed an organization that could be described as compound node where the PNS-CNS demarcation line runs across the nodal axon segment in the midst of the node gap. Branching at th borderline nodes was a very rare phenomenon. The length of the nodal axon segment, the nodal axon membrane area and the amount of paranodal mitochondria were similar to earlier presented data of feline ventral root fibres. The hypothesis that the elements of the paranodal pparatus of PNS fibres compensates for the lack in the PNS f nodally connected astrocytes is presented and discussed. A small umber of the fibres lacked a borderline node. In these cases the CNS myelin sheath projected uninterrupted for hundreds of μm into the PNS compartment where it was enclosed by a Schwann cell and PNS myelin. This rare arrangement was referred to as a transitional internode.

Somatic myelinated nerve fibres of the mammalian peripheral nervous system (PNS) either originate or terminate in the central nervous system (CNS). Hence, they all have to cross the PNS-CNS borderline. This passage implies profound re-arrangements of the axonal surroundings. In the PNS one Schwann cell attends a restricted segment of one single axon and supplies the internodal ends with the components of the the paranodal apparatus: mitochondria and node-gap microvilli (for references see Landon and Williams, 1963, Williams and Landon, 1963; Berthold, 1968b· Bischoff and Thomas, 1975, Landon and Hall 1976 In the CNS one oligodendrocyte gives rise to several internodal myelin sheath segments belonging to different axons. There are no paranodal accumulations of mitochondria, and node-gap microvilli are few and poorly developed (see review by Bunge, 1968 Hildebrand, 1971). Thus one of the most intriguing features of PNS-fibre organization — the paranodal apparatus — appears to be virtually absent in the CNS.

Myelinated fibres in the transitional region

Available data indicate that the transition from one nervous tissue compartment to the other as seen in a myelinated fibre takes place at a node of Ranvier — the borderline node — where an internode of PNS origin meets one of CNS origin (Foncin, 1961 Friede 1961 Maxwell *et al.*, 1969) Whether the borderline node consists of a typical PNS half and a typical CNS half or shows a preponderance of either pattern or whether it possesses certain unique features is unknown. In connection with the PNS to CNS transition of myelinated fibres the current literature supplies conflicting data as regards such functionally significant parameters as axon size, myelin sheath thickness and degree of fibre branching (Maxwell *et al.*, 1969· Ross and Burkel, 1971 Fraher 1976 see review by Gamble, 1976) In no case have comparisons been performed at CNS and PNS levels of the same axon. A direct serial section analysis of the myelinated fibres as they appear in the transitional region itself would then obviously provide straight forward information on many an obscure issue.

The present study deals in qualitative and quantitative terms with the myelinated nerve fibres of the dorsal root S_1 transitional region (TR) as observed light and electron-microscopically in the adult cat (see Berthold and Carlstedt, 1977). In short, the results — intended to serve as a standard for future work on the development of the TR — show that there is no difference in axon size on the two sides of the borderline node, that a recalculation of axon sizes and myelin sheath thickness to the native condition gives similar CNS and PNS values, that besides borderline nodes there are also transitional internodes and that the organization of the borderline node discloses unique features, some of which might be taken to support the idea that the paranodal apparatus might be the PNS counterpart of astrocytes related to the nodes of Ranvier in the CNS.

MATERIAL AND METHODS

Eight adult cats were anaesthetized intraperitoneally with pentobarbital, 40 mg/kg BW and artificially ventilated with 100% oxygen The lower spinal cord was fixed by vascular perfusion of a solution containing 5% glutaraldehyd for 20 minutes. The specimens, consisting of th segment S_1 and its dorsal roots, were removed by a laminectomy which required 10-15 minutes f preparative work (see Carlstedt, 1977).

Teased specimens

Specimens of 4 cats were after perfusion fixation rinsed for a short time in the buffer and the roots were carefully teased under a dissecting microscope into individual fibres.

Estimation of nternodal length Teased specimens were osmicated for 2 hours in 2% OsO in 300 mOsm Millonig buffer and mounted in glycerine on glass slides. In order to avoid pressure on the specimens strips of adhesive tape 50 μm thick were placed on the glass slides to support the cover glass Internodal length and fibre diameter were determined in the proximally situated peripheral internodes and in internodes of the mid part of the root, using a scale in the eyepiece f a Leitz light microscope

$NADH_2$-diaphorase activity Teased specimens were incubated at 37°C for 30 min tes in a medium containing NADH as a substrate and Nitro BT as reagent (Becker *et al.*, 1960· Berthold and Skoglund, 1967). Specimens incubated in a medium without substrate served as controls. After incubation the specimens were briefly rinsed in the Veronal buffer mounted in glycerine on glass slides and examined and photographed in Leitz photomicroscope.

Vestopal embedded specimens

Specimens of 4 cats were processed according to a method described in detail elsewhere (see Carlstedt, 1977). The specimens were, after postfixation, osmication and Vestopal-embedding trimmed iteratively into 30 µm high mesas using a LKB 11800 Pyramitome and then sectioned on LKB 4800 Ultrotome. The specimens were carefully orientated for cross sectioning so that the nerve fibres were cut perpendicularly to their longitudinal axis and sectioned in the distal to proximal direction. Longitudinal sections parallel to the longitudinal axis of the nerve fibres were obtained by sectioning the rootlets i the dorsoventral direction.

Three series f consecutive ultra thin cross-sections (average thickness 700 Å) were cut comprising 10,000, 6000 5,500 sections, respectively and four series of longitudinal sections of 200-1000 consecutive sections each. The average section thickness which was obtained from the ratio between mesa hight and the number of sections, varied between 600-800 Å. The loss of sections was less than 1%. The sections were placed on one hole copper grids coated with a carbon-stabilized formvar film After double staining with uranyl acetate (Brody, 1959) and lead citrate (Reynolds, 1963) the sections were examined in a Philips EM 300 electron microscope operated t 80 kV and equipped with a 30 µm objective aperture.

Sections for lightmicroscopy (0,5 µm thick) were placed on glass slides and stained with alkaline toluidine blue, examined, and photographed in a Leitz photomicroscope.

Quantitative estimations

Each cross-section series was photographed at consecutive 10 µm intervals and the myelinated fibres were identified and marked out by numbers on the photocopies. Proper identification was ascertained by the checking of intervening sections in the EM.

The *myelinated fibres were classified* as large, type I (D 12µm), medium, type II (6 D 12 µm) and small, type III (D 6 µm) fibres. Since this classification corresponds to the three groups of afferent fibres as separated by Lloyd (1943) with regard to conduction velocity *the proper classification of the present m terial can be performed first after the fiber size of the unprocessed fibres* (D native fibre size) has been calculated. This was done here by adding together the axon diameter (d), compensated for by a factor of shrinkage 1 1 (see Berthold, 1968b, Carlstedt, 1977) and the double myelin sheath thickness as obtained from the umber of myelin lamellae (n) times the original myelin period given as 180 Å (see Finean, 1960) and 160 Å (see Hildebrand and Müller 1974) in the PNS and CNS respectively Thus D_{PNS} 1 1 d_{PNS} 2x180 n_{PNS}, D_{CNS} 1.1 d_{CNS} 2x160 n_{CNS}. The PNS and the CNS size of one and the same fibre were estimated 100 µm distally and proximally to the PNS-CNS borderline respectively

The axon size ( ) was determined both as the length f the axon circumference ($a_{circ.}$) measured with map distance reader and as the size f the axon cross-section area (a_{area}) as estimated by planimetry A third estimate of axon size, a_{ratio} was obtained by the

size of cross section area
ratio
length of circumference

Th number of myelin lamellae was estimated from the number of major dense lines in each sheath The myelin period was measured as the mean distance between the midpoints of 10 successive major dense lines. The measurements were performed in regions of the myelin sheaths where the lameller pattern was regular nd distinct and where the course f the lamellae was parallel to the direction of sectioning Sharply curved parts of the sheaths were avoided (cf Karlsson 1966).

The hypothesis that there is a difference i fibre size between the investigated PNS and CNS levels was tested by an analysi of variance. First, the difference in axon size was tested using each of the three -estimates ($a_{circ.}$, a rea and a_{ratio}) separately Then the native calculated D-values were tested once, using the a-estimate which according to the analysis of variance showed the smallest CV value. Not surprisingly this turned out to be the ratio which has the higher information content. The analysis of variance was performed with the aid of computer (IBM 370/155) and a standard statistical programme

The umber *of Schwann cell mitochondria* was estimated by counting on photocopies of different paranodal and internodal levels (see Fig 6 and Table III). The n mber of mitochondria wa then plotted to scale in diagram with ref rence to the distance from the borderline node. Since the size of the area between the plotted lin and the base line expresses the amount of mitochondria given in arbitrary units, this latter parameter was estimated planimetry These estimations were performed on 27 fibres (3 fibres of each type animal).

Myelinated fibres in th transitional re

The *length of the nodal axon segment* (nl) was measured as the length of the undercoated axon membrane as seen in series of longitudinal sections. The parameter is given as the mean value of six measurements performed on each side of the axon in three sections: one median section and two paramedian sections located symmetrically between the median section and the axon perimeter In the same way the mean values of the length of the terminal PNS and CNS myelin sheath segments were also estimated. The nodal axon diameter (nd) was measured in the median section and corrected by the shrinkage factor of 1 1 Assuming that the nodal axon segment is a cylinder its membrane area (A) is very simple to calculate, viz. A nd π nl. These estimations were performed in 18 fibres (2 fibres f each type in each of 3 animals).

RESULTS

Internodal length

The internodal length measured 200-1000 μm in PNS borderline internodes. Halfway between the spinal cord and the spinal ganglion the internodes measured 300-1500 μm. At both levels internodal length was linearly correlated to fibre size with a correlation coefficient of 0.95 (Fig 1) In serveral cases it was noted that the internodal length along one and the same fibre increased gradually in the proximal to the distal direction as seen from the PNS-CNS borderline and for a distance corresponding to 3-4 internodes

Distribution of NADH$_2$-diaphorase activity

The distribution of the enzyme activity in the PNS compartment of the TR corresponded, with the exception of the PNS borderline paranode, to that given elsewhere for feline dorsal root fibres (Berthold and Skoglund, 1967 Berthold, 1968a). PNS borderline internodes thus revealed the rather stereotype pattern of a enzyme activity concentrated at the Schwann cell perikaryon, extending proximally and distally like delicate longitudinal stripes that broadened paranodally and terminated, still arranged like stripes, close to the node (Fig 7b). At PNS-borderline paranodes, however the bluish-black stripes coalesced and lost their identity some 5-10 μm away from the node. This gave the picture (Fig. 7a) of paranodal bulbs that contained irregularly-shaped pools of enzyme activity that extended up to and sometimes covered the node gap

Proximally to the borderline node the intensity of the staining was low and its distribution was uncharacteristic.

All controls were negative.

Fibre parameters on the two sides of the PNS-CNS borderline

Axon diameter The analysis of variance (Table 1) showed a low significance (p 0.05) with regard to a PNS-CNS difference in axon size as estimated from the $a_{circ.}$ and the a_{area} In the case of the a_{ratio} there was no difference between PNS and CNS size. A comparison between the three a-variables using the coefficient of variation (CV Table 1) showed that the a_{ratio} had a smaller CV and hence was the test variable to be preferred to $a_{circ.}$ and $a_{area.}$

Myelin lamellae In peripheral myelin sheaths the number of myelin lamellae varied between 20 and 130 (Fig ..) and in the central myelin sheaths between 10 and 180 (Fig 3). There was a more or less linear relationship between number of lamellae and axon size in small fibres where the number of lamellae varied between 20-100 in the PNS and between 10-90 in the CNS. Larger fibres contained a highly variable number of lamellae.

When comparing the number of lamellae in the peripheral and the central myelin sheath on the same axon (Fig 4) it was noticed that the smallest fibres (small group III fibres 4 µm) had fewer lamellae in the CNS than in the PNS and that the situation was the reverse in medium and large fibres. The PNS myelin period (Table II) was 120 Å (± 5) in all fibres alike The CNS myelin period varied between 81 and 117 Å, being inversely related to fibre size (cf. Hildebrand, 1972).

Fiber size. Recalculation of myelin sheath thickness and of axon size to the native condition enabled the whole fibre size (D) to be estimated. Fig 5 shows that the PNS D-values of the smallest fibres (4 µm) are larger than the corresponding CNS D-values Fibres more than 4 µm in size show a more or less contrary situation. As seen in Table II the mean values for the whole fibre population with regard to the PNS and CNS D-values indicated that the CNS part of a fibre might be somewhat thicker than its PNS part. This difference is also evident when using the analysis of variance (Table I).

The borderline segment

This part of a myelinated fibre found at the crossing of the PNS-CNS borderline consisted in the vast majority of fibres of a PNS (distal) borderline paranode, a CNS (proximal) borderline paranode and, an intervening borderline node of Ranvier A few fibres lacked a borderline node in which cases an uninterrupted CNS myelin sheath crossed the borderline. This arrangement was referred to as a transitional internode.

PNS borderline paranodes In all but the smallest fibres (4 µm) the paranodes were characterized by a high content of Schwann cell mitochondria (Figs. 6 8b 12) and by longitudinal myelin sheath crests (Fig 8a). The smallest fibres seemed to lack a paranodal Schwann cell mitochondrion accumulation (see below and Table III) The length of the paranodes varied from about 10 µm in small fibres to about 40 µm in large ones.

The paranodes together with a surrounding narrow *cul de sac* of the endoneural space, were as a rule isolated from neighbouring fibres inside a holster of closely-packed astrocytic processes (Figs. 8a, 10a) (cf. Berthold and Carlstedt, 1977). The processes were part of the glial fringe and derived from the astrocytic perikarya of the mantle zone. The borderline node was situated at the bottom (proximal end) of the endoneural *cul de sac*. The larger the fibre the longer (or deeper) was the holster its length varying from 20 down to only a few µm. The lumen of a holster adapted its shape to that of the enclosed paranode. The intervening endoneural gap measured about one µm in width (Figs. 8a, 10a). This arrangement restricted or eliminated the perinodal space, the latter being more or less replaced by the feltwork of astrocytic processes.

In contrast to ordinary PNS paranodes borderline paranodes showed myelin crests that disappeared several (5-10) µm form the node (Fig 8b). Proximally to this level the cords of Schwann cell cytoplasm of more distal levels joined and often formed a more or less common cytoplasmic compartment very rich in mitochondria (Figs. 8b, 12) In several cases this compartment extended close to the node. At some nodes it covered part of the node gap (Fig 12) In the latter cases Schwann cell microvilli projected from the mitochondrion-rich compartment straight into the node gap

The highest numbers of paranodal Schwann cell mitochondria were noted in cross sections 3-7 µm from the node gap (Fig 6). The countings were here about 300 200 and 30 mitochondria per cross section in average large, medium and small fibres respectively The amount of paranodal mitochondria, as expressed in arbitrary units (Table III and Fig 6) was in the same fibre types 100-200 40-80 and 1 10 respectively The smallest fibres showed no clear accumulation of paranodal mitochondria. In addition to mitochondria, the paranodal Schwann cell cytoplasm

contained electron-dense granules 200-300 Å in size (presumably glycogen), myelinoid bodies ?-5 μm in diameter and bodies 0.5-2 μm in diameter consisting of a homogeneous low-density material lacking a limiting membrane presumably lipid droplets (cf. Berthold, 1968b)

CNS paranodes. The contour of cross cut paranodal CNS myelin sheaths was rounded and lacked myelin crests (Fig 8d). The paranodes pierced the mantle zone and were surrounded by astrocytic processes and astrocytic perikarya (Figs. 8, 9). Both the processes and the perikarya were rich in gliosomes. Myelinoid bodies up to 10 μm in size were common outside the CNS paranodes (Fig 8d) (cf. Berthold and Carlstedt, 1977)

Borderline nodes The node-gap content was as a rule disarranged to some extent by tiny empty-looking torn spaces. The gaps contained in their distal part a corona of closely packed Schwann cell microvilli and in their proximal part some irregularly-shaped astrocytic extensions (Figs 8c, 9 10a, b). The processes were surrounded by a finely granular node gap matrix substance. The two kinds of processes radiated towards the nodal axon plasma membrane, terminated 50-100 Å away from it and occupied each about half of its area (Table IV)

The axon diameter was reduced to less than half of its internodal size in those segments where the PNS-CNS-myelin sheaths terminated and in the intervening nodal segment. The cross-section contour was almost circular The constricted axon segment contained tubules, filaments, scattered mitochondria and vesicles. The axon segment related to the PNS-myelin sheath termination, and adjacent parts of the nodal axon segment contained an accumulation of 200-300 Å-large electron dense granules (Fig. 9) (cf. Berthold, 1968b). The length of the nodal axon segment was about 1 μm in all fibres and its membrane area measured 11 20 7-9 and 2.5-5 μm in large, medium and small fibres respectively (Table IV)

The borderline segment of the smallest (4 μm) fibres was of a similar general outline to that described above. The node gap appeared however more roomy and contained broad Schwann cell and astrocytic processes (Fig 11). The peripheral paranode showed few and low myelin crests and the outer Schwann cell compartment contained comparatively few mitochondria (Table III, Fig 6).

In two cases out of about 1000 investigated fibres axon bifurcation was noted at the borderline node (Fig 13) Apart from the bifurcation the borderline segments of such fibres were morphologically similar to ordinary ones. The branches were thinner than the parent fibre.

Transitional internodes Each rootlet contained superficially in its PNS compartment one or two medium sized axons (i.e. less than 1% of the myelinated fibres) which for about 150-200 μm were equipped with both a central and a peripheral myelin sheath (Fig 14a) The CNS-myelin sheath was telescoped inside the PNS-myelin sheath, both surrounding the axon.

Such aberrant fibres consisted of the following concentric layers as traced in the centripetal direction basement membrane outer cytoplasmic compartment of the Schwann cell, peripheral myelin sheath inner Schwann cell compartment, astrocyte cytoplasm, central myelin sheath a thin, presumably oligodendrocyte cytoplasmic layer and a central axon (Figs 14a, b) The plasma membranes of the inner Schwann cell compartment and the astrocyte cytoplasm were separated by a space 200-300 Å wide. These two cytoplasmic compartments were missing at some sites and the central and peripheral myelin sheaths apposed each other forming a myelin-sheath intraperiod line at the zone of contact (Fig. 14c).

The peripheral myelin sheath terminated at the surface of the mantle zone. It formed cytoplasmic pockets which were attached to the underlying central myelin sheath (Fig 14d). The Schwann cell contained in this position more mitochondria than were observed internodally and the proximal end of the outer Schwann cell

compartment formed a few short and broad processes. Fissures, both in the myelin sheath and in the surrounding glia were common at the borderline passage of transitional internodes.

DISCUSSION

The problems of obtaining ultrastructurally well-preserved myelinated nerve fibres at their passage between the PNS and CNS compartments in feline S_1 dorsal rootlets are great and have been dealt with in a preceding communication (Carlstedt, 1977). Although that report showed that it was possible to improve the preservation of the myelin sheaths and the glia considerably none of the preparatory methods tested also gave a fully satisfactory preservation of the node-gap ultrastructure. Torn spaces and disarranged Schwann cell and glial processes were constantly noted at least at some sites in the gaps of large and medium-sized fibres. Hence, estimations of node-gap volume and the number and the area of the node gap processes cannot be performed The preservation outside the node gaps may on the other hand, be regarded as satisfactory (cf Carlstedt, 1977) and the data obtained from measurements of such parameters as myelin-sheath period, axon size, length of nodal axon segment and so forth (see under Material and Methods) are considered reliable enough to be used below without further comment.

Axon size. Axon size (a) was estimated in three ways: a_{circ}, a_{area} and a_{ratio} as the length of the axon circumference, as the axon cross-section area and as the area to circumference ratio respectively Using these three a-variables the hypotheses concerning a difference between aPNS and aCNS of the same axons was tested in an analysis of variance. Even if the cats are considered as fixed effects (Table I) there is very little support of a difference (p 0.05) of a_{circ} and a_{area} and there is no significant difference when testing a_{ratio}. As shown by the coefficients of variation (Table I) a_{ratio} shows the smallest value and is the variable to be preferred of the three tested. Hence our data do not seem to support the idea (see Tarlov 1937 Gamble, 1976) that passage into the CNS means a decreased axon size. Instead they indicate that axon size remains unchanged.

Myelin sheath and whole fibre diameter The plotting of PNS-axon diameter (from a_{ratio}) against the number of surrounding myelin lamellae gave a more or less rectilinear relationship in axons 1-4 µm in size where the number of lamellae varied between 20-100. Rectilinearity was lost in the group of larger axons 4-12 µm in size where several fibres carried about the same number of about a hundred lamellae in spite of a threefold variation in axon diameter A maximum number of 130 lamellae was noted outside two axons 10 6 and 12.6 µm thick. In the CNS a rough rectilinearity was present in axons up to 3 µm thick carrying 10-90 lamellae. CNS axons more than 4-5 µm thick were equipped with a highly variable number of lamellae, ranging between 100-160 A maximum of 170-180 lamellae was noted outside the largest axons (10-13 µm). Thus it appears as if a myelin sheath thickness of 100-200 lamellae would give sufficient insulation in both PNS and CNS axons which are 4-13 µm in diameter

In a series of works on mouse and rat peripheral nerves using electron microscopy Friede and coworkers (1967 1968, 1972) found that axon size and number of myelin lamella are rectilinearly related throughout the fibre spectrum. Superficially this is in contrast to the situation in feline dorsal roots. However since in the mouse 100 lamellae is about the maximum number found outside the largest axons some 6-7 µm thick, there seems to be fairly good agreement between mouse fibres in general and small to medium-sized feline axons. The larger axons which in the cat carry highly variable number of lamellae and invalidate rectilinearity are simply not present in the mouse.

Fibres belonging to group I and II (> 6 μm) carried an average of 20-30 additional CNS lamellae. In small group III fibres (< 4 μm) transition into the CNS meant a loss of 10-15 lamellae corresponding roughly to 25% of the PNS number. The larger group III fibres (fibre size 4-6 μm) did on average acquire some additional lamella in the CNS. Hence it appears as if axons more than 2 μm in size roughly increased their number of myelin lamellae by an average of 20-25% as they reached into the CNS. Whether the higher number of myelin lamella along the CNS part of an axon is significant in terms of impulse transmission remains to be shown. In fact, several fibres showed no CNS-PNS lamellar difference or even a negative one.

The common preparative procedure used in electron microscopy includes dehydration and plastic embedding, during which the interlamellar distance of myelin shrinks by about 40-50% (from 160 Å to 80 Å) and about 30% (from 180 Å to 120 Å) in the CNS and the PNS, respectively (Finean, 1960; Karlsson, 1966; *Hildebrand and Müller 1974). So in the embedded condition there are in fact few* fibres that reveal a CNS myelin layer that is thicker than the PNS one (cf. Table II). The difference between the CNS and the PNS in interlamellar distance of fresh myelin means that the CNS part of a fibre has to carry 13% additional lamellae if the CNS and PNS-myelin-sheath thicknesses are to be equal in the unfixed condition. In larger fibres where the preparative CNS myelin shrinkage is 50%, this means that a PNS-myelin sheath of 100 lamellae which when unfixed is of the same thickness as a 113-lamellar CNS sheath will after processing appear to be about 25% thicker. Thus the CNS nyelin in question must, if it in the embedded condition is to be of a thickness equal to that of its PNS counterpart consist of 150 lamellae. It is then obvious that CNS and PNS myelin sheath thicknesses cannot be compared unless they are recalculated to the unfixed condition. Maxwell *et al.* (1969) who stated in general terms, that myelin sheath thickness decreases as the fibres enter the CNS based their conclusions on very small monkey fibres. As is also found in the cat (Fig. 4) very small fibres (4 μm) decreased in myelin sheath thickness as they reached into the CNS. Recalculated values for the whole fiber population indicated that CNS D-values may be somewhat larger than the corresponding PNS values in fibres larger than 4 μm (Fig 5, Table II). Comparisons between dorsal root and spinal-cord calibre spectra (among others see Skoglund and Romero, 1965; Hildebrand and Skoglund, 1971 a) show however that the group of large CNS fibres definitely are thinner than the PNS ones. Conduction velocity is also reported to slow down after passage into the CNS (Lloyd and McIntyre, 1950; Collins and Randt, 1961; Petit and Burgess, 1968; Brown, 1968). These differences between CNS and PNS fibres are currently explained by fibre branching at the CNS entrance. Yet since branching was an exceptional observation in feline dorsal root borderline nodes it must in the cat be a phenomenon of more centrally located nodes.

Internodal length. The rectilinear increase in internodal length with increasing fibre size is well known and is considered to be of the same magnitude in CNS and PNS fibres alike (Vizoso and Young 1948; Thomas and Young 1949; Ohlrich and McDonald, 1974). The internodes found in the PNS compartment of the transitional region, i.e. the most proximally located PNS internode of a fibre, revealed a length 30% shorter than expected from fibre size and the situation noted more distally in the root (see Fig 1). In most fibres the decrease in internodal length was not sudden but gradual, comprising the last 2-4 PNS internodes. Provided that other fibre factors remain equal, a gradually decreased internodal length should mean a gradually increased depolarizing nodal current and a comparatively most advantageous situation as regards the safety factor in that part of the fibre where the passage into the CNS takes place (Tasaki, 1953). This brings up the issue of the borderline node.

The borderline node As already pointed out in the introduction, there are striking differences between the structural organization of CNS and PNS node-paranode regions and it is questionable whether the term paranodal apparatus is at all relevant in the CNS. Two of the three most characteristic features of the paranodal apparatus are simply not found in the CNS and the third is only indicated. Thus paranodal myelin-sheath crenation and mitochondrion accumulation have proven to be exclusively a PNS specialization (cf. Berthold, 1968 b; Hildebrand, 1971) The node-gap corona of densely packed Schwann cell microvilli that interconnects the immediate vicinity of the nodal axon membrane with the mitochondrion pools is in the CNS replaced by a few rudimentary-looking astrocytic processes. Moreover the node gaps of the PNS are surrounded by a comparatively voluminous extracellular space: the endoneural room In the CNS the extracellular space is very much restricted and forms a labyrinthine system of extremely flat spaces (cf. Bunge, 1968) Features that, on the other hand appear to be common to both CNS and PNS nodes are a coated nodal-axon membrane which varies from 3 μm to 25 μm with increasing fibre size (cf. calculations by Berthold, 1968 b; Hildebrand, 1971) a node-gap substance that gives a positive reaction to tests on acid mucopolysaccarides, and node-gap walls formed by myelin-sheath terminal cytoplasmic pockets (Elfvin, 1961 Berthold, 1968 b; Langley and Landon, 1969; Hildebrand, 1971 Hildebrand and Skoglund 1971 b). Hence, since the principle of impulse conduction are considered to be the same in the CNS and PNS (Rasminsky and Sears, 1972) available data seem to imply that the elaborate paranodal apparatus should actually be insignificant in terms of impulse generation and propagation.

The present results indicate that the borderline node-paranode region in several respects resembles what might be called a PNS-CNS compound node. This is clearly demonstrated in series of sections where the separation line runs in the middle of a node gap which in its peripheral half lodges a microvilli corona that more distally is transformed into a mitochondrion-rich paranodal Schwann cell compartment and which in its central half reveals a few blunt astrocytic processes The size and shape of the nodal axon resembled those of genuine PNS and CNS nodes.

In contrast to ordinary peripheral paranodes, those of the borderline segment were holstered and isolated from their neighbours by astrocytic processes their myelin crests were less developed close to the node and paranodal accumulations of Schwann cell mitochondria extended very close to the node gaps, at some sites even covering parts of the gap (see Fig 12) Beside its relation to astrocytic perikarya (see below) the CNS-borderline paranode appeared similar to paranodes as seen elsewhere in white matter Taking into account these differences as well as the short length of the adjoining PNS paranode, it can be concluded that the borderline node of Ranvier is a compound PNS-CNS node with some unique features.

Calculations of the amount of mitochondria in large (group I) borderline PNS-paranodes and of ordinary alpha-fibre paranodes of a corresponding size show roughly equal values (Berthold, 1968b) Comparisons with regard to group II and III fibres cannot be performed because of the absence of reference data. The variation with fibre size in the amount of mitochondria of the PNS borderline paranodes is in line with the previously made suggestion that A fibres constitute two main groups, one comprising small fibres with a poorly developed paranodal apparatus (here small — D 4 μm — group III fibres) and the other the larger fibres with well developed apparatus where, for instance, the amount of mitochondria increases rapidly with fibre size (here group I and II fibres) (cf. Berthold, 1968b; Phillips *et al.*, 1972).

A comparatively high number of astrocytic perikarya occurred in close relation to the paranodes of the mantle zone. As pointed out in a previous communication on the transitional region (Berthold and Carlstedt, 1977), the mantle-zone corresponds to the external glial limiting membrane which is formed by a complex feltwork of astrocytes and it was calculated that the occurrence of

Table II

Myelinated fibre parameters as estimated [in] the same crossectioned dorsal root fibres on the two sides of the borderline node.[1)]

Animal	Fibre type	Axon diameter calculated from circumference, Mean value µm		Axon diameter calculated from area, Mean value µm		Axon diameter calculated from area/circumference, Mean value, µm		No. of myelin lamellae, Mean value (SD)		Myelin period, Mean value (SD) Å		Calculated fixed myelin sheath thickness, Mean value (SD) µm		Calculated native myelin sheath thickness, Mean value (SD) µm		Calculated native total fibre diameter, Mean value, µm	
		PNS	CNS	PNS	CNS	PNS	CNS	PNS	CNS	PNS	CNS	PNS	CNS	PNS	CNS	PNS	CNS
	I	12.73	12.44	11.68	11.43	10.74	10.62	106.80 (34.38)	140.80 (22.72)	123.30 (1.16)	83.6 (2.76)	1.43 (0.11)	1.77 (0.21)	2.08 (0.16)	2.26 (0.36)	18.99	16.60
K 1027	II	6.69	6.20	5.86	5.53	6.21	4.99	88.64 (9.18)	118.62 (40.69)	108.27 (32.66)	86.09 (2.86)	1.07 (0.13)	1.12 (0.17)	1.62 (0.17)	2.06 (0.33)	8.96	10.06
	III	3.19	2.94	2.42	2.20	1.94	1.60	41.27 (8.01)	33.11 (21.79)	120.22 (6.12)	111.44 (33.06)	0.49 (0.09)	0.32 (0.18)	0.74 (0.14)	0.64 (0.36)	3.49	3.17
	I	12.00	11.96	11.18	10.97	9.92	9.44	117.28 (8.21)	148.18 (19.00)	122.18 (6.67)	90.82 (4.12)	1.43 (0.12)	1.34 (0.13)	2.11 (0.15)	2.37 (0.31)	16.13	16.72
K 1039	II	7.36	7.67	6.40	6.81	6.22	6.27	102.33 (9.07)	134.33 (18.20)	123.89 (14.28)	91.76 (2.71)	1.26 (0.11)	1.23 (0.17)	1.84 (0.16)	2.16 (0.31)	9.43	10.86
	III	3.34	3.67	2.59	2.41	1.72	1.82	43.88 (11.34)	64.20 (28.60)	123.70 (1.77)	103.3 (6.34)	0.86 (0.37)	0.60 (0.23)	0.80 (0.19)	0.86 (0.42)	3.49	3.96
	I	12.36	11.78	10.47	10.17	6.66	8.81	118.10 (3.70)	120.60 (10.64)	116.1 (0.57)	90.9 (4.07)	1.33 (0.04)	1.10 (0.08)	2.08 (0.08)	1.93 (0.17)	13.95	13.98
K 1040	II	8.33	7.84	6.78	6.69	8.66	6.60	102.00 (7.69)	110.19 (17.29)	117.4 (6.18)	91.4 (3.24)	1.20 (0.12)	1.00 (0.14)	1.84 (0.14)	1.71 (0.28)	9.79	10.22
	III	2.38	2.07	1.73	1.64	1.27	1.31	32.7 (6.52)	24.4 (8.28)	118.7 (4.32)	101.4 (8.24)	0.40 (0.07)	0.26 (0.07)	0.69 (0.12)	0.40 (0.13)	2.66	2.34
K 1027	I	12.36	12.07	11.11	10.60	9.84	9.69	113.07 (20.20)	134.43 (21.24)	120.2 (6.31)	88.43 (4.43)	1.40 (0.11)	1.21 (0.18)	2.09 (0.13)	2.18 (0.34)	16.03	16.45
K 1039	II	7.53	7.28	6.36	6.31	5.33	5.40	97.97 (10.38)	121.07 (21.13)	116.86 (6.78)	90.42 (3.42)	1.17 (0.16)	1.11 (0.17)	1.77 (0.16)	1.97 (0.09)	9.38	10.28
K 1040	III	2.98	2.71	2.24	2.16	1.83	1.64	38.77 (9.76)	37.40 (23.47)	120.87 (4.68)	106.38 (7.42)	0.84 (1.11)	0.36 (0.20)	0.71 (0.18)	0.61 (0.38)	3.17	3.16

1) The crude data from 80 myelinated fibres on the two sides of the borderline node will be supplied on request.

Table III

Numerical estimations of mitochondria in the PNS borderline paranodes

Animal	Fibre type	Amount of paranodal mitochondria in arbitrary units (see Fig. 6)	Highest number of mitochondria in the paranode	Distance from the borderline node of the highest number f mito-chondria (μm)	Number of mitocondria 100 μm distal to the border line node
		196	264	10	12
	I	163	347	4	12
		169	320	4	11
		44	168	4	6
K 1027	II	55	217	4	6
		49	182	4	4
		9	81	2	2
	III	3	17	4	4
		1	6	4	2
		100	254	4	8
	I	147	282	7	22
		77	227	4	14
		61	114	16	10
K 1039	II	47	146	4	6
		34	202	4	10
		2	60	4	4
	III	2	60	4	10
		1	4	4	2
		178	271	10	38
	I	130	220	6	14
		103	260	4	26
		61	166	10	34
K 1040	II	32	110	4	4
		68	214	7	10
	III	3	12	4	4
		1	39	4	4
		2	61	4	6

Myelinated fibres in the transitional region

Table 11

Myelinated fibre parameters as estimated in the same crossectioned dorsal root fibres on the two sides of the borderline node.[1)]

Animal	Fibre type	Axon diameter calculated from circumference, Mean value, µm PNS	Axon diameter calculated from circumference, Mean value, µm CNS	Axon diameter calculated from area, Mean value, µm PNS	Axon diameter calculated from area, Mean value, µm CNS	Axon diameter calculated from area/circumference, Mean value, µm PNS	Axon diameter calculated from area/circumference, Mean value, µm CNS	No. of myelin lamellae, Mean value (SD) PNS	No. of myelin lamellae, Mean value (SD) CNS	Myelin period, Mean value (SD) Å PNS	Myelin period, Mean value (SD) Å CNS	Calculated fixed myelin sheath thickness, Mean value (SD) µm PNS	Calculated fixed myelin sheath thickness, Mean value (SD) µm CNS	Calculated native myelin sheath thickness, Mean value (SD) µm PNS	Calculated native myelin sheath thickness, Mean value (SD) µm CNS	Calculated native total fibre diameter, Mean value, µm PNS	Calculated native total fibre diameter, Mean value, µm CNS
K 1027	I	12.73	12.44	11.08	11.43	10.74	10.52	105.80 (34.38)	140.80 (22.72)	123.30 (1.18)	93.6 (2.78)	1.43 (0.11)	1.77 (0.21)	2.08 (0.16)	2.28 (0.36)	15.98	16.60
K 1027	II	6.80	8.20	5.86	6.53	8.21	4.90	89.64 (9.16)	118.82 (40.88)	108.27 (32.68)	88.09 (2.86)	1.07 (0.13)	1.12 (0.17)	1.82 (0.17)	2.06 (0.33)	8.96	10.06
K 1027	III	3.19	2.94	2.42	2.20	1.94	1.80	41.22 (8.01)	33.11 (21.78)	120.32 (8.12)	111.44 (33.08)	0.49 (0.09)	0.32 (0.19)	0.74 (0.14)	0.54 (0.36)	3.48	3.17
K 1038	I	12.00	11.96	11.16	10.87	9.92	9.44	117.28 (8.21)	148.18 (19.00)	122.18 (6.57)	90.62 (4.12)	1.43 (0.12)	1.34 (0.13)	2.11 (0.16)	2.37 (0.31)	16.13	15.72
K 1038	II	7.38	7.87	6.40	6.81	6.22	6.27	102.33 (9.07)	134.33 (19.20)	123.89 (14.25)	91.78 (2.71)	1.26 (0.11)	1.23 (0.17)	1.84 (0.16)	2.18 (0.31)	9.43	10.96
K 1038	III	3.34	3.57	2.89	2.41	1.72	1.62	43.88 (11.34)	64.20 (26.63)	121.70 (1.77)	103.3 (6.34)	0.66 (0.37)	0.80 (0.23)	0.80 (0.19)	0.88 (0.42)	3.40	3.96
K 1040	I	12.38	11.78	10.47	10.17	8.68	8.81	116.10 (3.70)	120.80 (10.64)	115.1 (0.87)	90.9 (4.07)	1.33 (0.04)	1.10 (0.08)	2.06 (0.09)	1.93 (0.17)	13.96	13.96
K 1040	II	8.33	7.84	6.78	6.68	5.56	5.60	102.00 (7.68)	110.10 (17.29)	117.4 (5.18)	91.4 (3.24)	1.20 (0.12)	1.00 (0.14)	1.84 (0.14)	1.71 (0.28)	9.79	10.22
K 1040	III	2.38	2.07	1.73	1.84	1.27	1.31	32.7 (6.62)	24.4 (8.25)	116.7 (4.32)	101.4 (8.24)	0.40 (0.07)	0.26 (0.07)	0.59 (0.12)	0.40 (0.13)	2.56	2.34
K 1027 K 1038 K 1040	I	12.36	12.07	11.11	10.80	9.84	9.59	113.07 (20.20)	134.43 (21.24)	120.2 (5.31)	88.43 (4.43)	1.40 (0.11)	1.21 (0.18)	2.09 (0.13)	3.18 (0.34)	16.03	16.48
K 1027 K 1038 K 1040	II	7.83	7.28	6.36	6.31	5.33	5.40	97.97 (10.39)	121.07 (21.13)	118.88 (6.78)	90.42 (3.42)	1.17 (0.18)	1.11 (0.17)	1.77 (0.18)	1.97 (0.06)	9.36	10.28
K 1027 K 1038 K 1040	III	2.98	2.71	2.24	2.18	1.63	1.64	38.77 (9.78)	37.40 (23.47)	120.87 (4.88)	106.38 (7.42)	0.84 (1.11)	0.38 (0.20)	0.71 (0.18)	0.61 (0.38)	3.17	3.18

1) The crude data from 90 myelinated fibres on the two sides of the borderline node will be supplied on request.

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LEGENDS TO FIGURES

Abbreviations used i Figs. and legends

A	astrocyte
Ax	Axon
B	Basement membrane
BN	borderline node of Ranvier
C	central myelinated fibre
CM	CNS myelin
E	endoneural space
EM	electron micrograph
G	gliosom
LM	light micrograph
MB	myelinoid body
N	- node of Ranvier
P	peripheral myelinated fibre
PM	PNS myelin
S	Schwann cell

Fig. 1 Internodal length and fiber diaméter in the S_1 dorsal root of four different cats as measured in the most proximally situated peripheral internode (DRP) and in internodes situated half way between the pinal cord and the dorsal root gangtion (DRM). The regression line for DRP is, y 40 749x 184 664 and for DRM, y 53.897x 354.007

Figs 2, 3 Number of myelin lamellae plotted against axon diameter as calculated from the ratio between axon area and length of circumference on the PNS (Fig 2) and CNS (Fig. 3) side of the transitional nodes in 90 fibres of three cats. The symbols refer to the different animals.

Fig. 4 Difference in number of myelin lamellae between the CNS and PNS parts of a fibre plotted against axon diameter (calculated from the ratio between axonal area and length of circumference). Same fiber population as Figs. 2 and 3.

Fig 5 Calculated native CNS fibre diameter (D_{CNS} plotted against calculated native PNS fibre diameter The solid line indicates where D_{CNS} D_{PNS} Same fibre population as in Figs. 2 and 3

Fig 6 Occurrence of Schwann cell mitochondria within 100 μm from the borderline node (NoR) in one group I fibre (D 15.7 μm) one group II fibre (D 9 μm) and two group III fibres (D 6 μm and 2.5 μm indicated by a single and a double hatch respectively). For comparison data of a large feline ventral root fibre (hatched line) was included (fibre No. 3 from Berthold, 1968b).

Fig. 7 a, b LM Teased S_1 dorsal rootlet. Incubation for $NADH_2$-tetrazolium reductase activity The pictures show the proximal (a) and the distal (b) ends of the PNS borderline internode of a large myelinated fibre. In contrast to the arrangements in the distal paranode (b) the bands of enzyme activity of the proximal borderline paranode coalesce into one pool (arrows) (x1300).

Fig 8 a-d EM. Cross-sections through different levels of the borderline segment.

) Section level 15 μm distally to borderlin node. Interwoven astrocytic processes of the glial fringe (GF) encloses the fibre with a minute concentric endoneural space (E) in a tube- or bolster-lik fashion. The myelin sheath is crenated. The accumulations of Schwann cell cytoplasm in the furrows between the myelin crests contains mitochondria and one myelinoid body (MB) (x4500).

b) Section level 3 μm distally to borderline node. The myelin sheath is of an ovoid outline and has started its uncoiling along the axon. The Schwann cell cytoplasm is accumulated to a large coherent compartment (S) loaded with mitochondria (cf. Fig 7a) (x6200).

Myelinated fibres in the transitional region

MATERIAL AND METHODS

Eight adult cats, body weight 3-5 kg were anaesthetized intraperitoneally with pentobarbital, 40 mg/kg BW and artificially ventilated by oxygen administrated through a tracheal cannula. The lumbosacral spinal cord and its roots were fixed by vascular perfusion with phosphate buffered 5% glutaraldehyde Dorsal rootlets belonging to the segment S_1 were carefully removed together with small pieces of the adjoining spinal cord white matter postfixed, osmicated dehydrated in acetone and embedded in Vestopal W (for details see Carlstedt, 1977)

The Vestopal-embedded specimens were before sectioning trimmed into the shape of a flat mesa using a LKB 11800 Pyramitome Ultra-thin cross-sections with an interference colour of gray to pale yellowish-white corresponding to a section thickness of about 500-1000 Å (see Peachey 1958) were cut on a LKB 4800 Ultrotome Three series of consecutive cross sections comprising 10,000 6000 and 5,500 section and four series of 200-1000 longitudinal sections were *made from the transitional region One series f 4,500 cross sections,* as well as random cross and longitudinal sections, were manuf ctured from the part f the root situated halfway between the ganglion and the spinal cord. The section thickness varied between 600-800 Å and was calculated by dividing the height of the mesa with the number f sections it had yielded. The loss of sections was less than 1%. The sections were placed on one-hole copper grids coated with a carbon-stabilized *formvar film, double-stained with uranyl acetate (Brody* 1959) and lead citrate (Reynolds 1963) and examined in a Philips 300 EM electronmicroscope.

The calibre spectra of unmyelinated fibres were estimated in the peripheral and the central nervous compartments f the same rootlet in three cats using a calibrated Zeiss TGZ3 particle size nalyser (cf Romero and Skoglund, 1965).

In the case f 27 fibres (9 of each animal) their sizes were measured at 10 µm intervals at 5 levels on each side of the PNS-CNS borderline in the most proximal part of the TR. For this photocopies of x 5000 magnification of the different levels and a scale of preformed circles were used. These fibres were also carefully examined in the electron microscope at 2 µm intervals to a distance of 100 µm proximally to the borderline. In this way the short internodal lengths observed along the CNS-extension of large C-fibres was estimated.

Schwann cell nuclear length and Schwann cell internuclear distance were estimated in 3 bundles of unmyelinated fibres from three cats. The measurements were performed at consecutive 5 µm intervals on photocopies (x 1000-2000). As a control intervening sections representing 2 µm intervals were checked in the electron microscope.

Terminology (see Berthold and Carlstedt, 1977). The transitional region (TR) is the first 1000 µm long segment in the proximal free part of a rootlet containing both CNS and PNS tissue A cross section through the TR reveals two concentric compartments: (1) the CNS compartment consisting of CNS tissue arranged in two zones (a) the core zone being situated in the centre of the rootlet and organized like a central fibre tract, (b) the mantle zone formed the PNS-CNS interface composed f astrocytes and nerve fibres (2) the PNS compartment containing PNS components and the astrocyte processes of the glial fringe the last emanating from the mantle zone.

RESULTS

Unmyelinated fibres distal to the transitional region

The unmyelinated axons constituted about 75% of the total number of the nerve fibres in the stereotype PNS part of S_1 dorsal rootlets The axons showed a round cross-section contour The axoplasm contained orangelles of the familiar appearance previously described by for instance, Elfvin (1958, 1968 see also Ochoa 1975, 1976.) The unmyelinated fibres were arranged in bundles connected to Schwann cells (Fig 1a) The bundles consisted as a mean value of 21 (SD 8, *n=30)fibres at the level of the Schwann cell nucleus. As a rule each fibre was* separated from adjacent ones by septa of Schwann cell cytoplasm All fibres of a

bundle could easily be followed in serial cross sections for up to some 200 μm without any major divergencies or shifts in position (size of observation field. 10×12 μm) (cf. Gasser 1955; Aguayo et al., 1976). Schwann cell internuclear distance was about 100 μm (cf. Gasser 1955; Peyronnard et al 1973 Aguayo et al. 1976).

Unmyelinated fibres in the PNS compartment of the TR

In this part of the rootlet a bundle of unmyelinated fibres consisted as a mean value of 9 (SD 4 n=30) fibres at the level of the Schwann cell nucleus. Close to the PNS-CNS borderline there was a pronounced exchange and criss crossing of fibres between the different bundles. This was illustrated by the failure to trace the fibres in a bundle for more than some 20 μm using an observation field 10×12 μm in size.

In nine bundles investigated, the mean distance from the PNS-CNS borderline to the most proximally situated Schwann cell nucleus was 23 μm (SD 16). The mean distance between successive Schwann cell nuclei along a bundle of unmyelinated fibres was 12 μm (SD 12) and the mean Schwann cell nucleus length 10 μm (SD 3) (see Fig 2)

The fibre spectrum analysis of three rootlets (Table I) showed a unimodal pattern of distribution with the peak at 0.5 μm. About 70% of the fibres measured 0.35-0.57 μm Some 5% were 0.8 μm or more in diameter The largest axons measured 1.2 μm. The maximum variation in individual fibre size with regard to the mean diameter was 38% (Table II).

Unmyelinated fibres in the CNS compartment of the TR.

In the CNS compartment of the rootlet the number of unmyelinated fibres was about 70% of the total number of nerve fibres. They were about 5% less than in the PNS part of the rootlet. The axons were of a somewhat angular outline and their axoplasm contained the same constituents as were noted in the PNS part of the fibres. The axons occurred in bundles consisting of 10-40 axons without intervening cytoplasmic septa (Fig 1b). In the mantle zone of the CNS compartment just proximally to the PNS-CNS borderline, the fibres occurred in bundles consisting of about 10 fibres. Here there was an extensive interchange of fibres between the different bundles. Thus a bundle of fibres existed as a unit only for some 10-20 μm (size of the observation field, 10×12 μm). More proximally in the core zone the thin bundles aggregated to form larger units consisting of up to 40 individual fibres.

The calibre spectrum analysis (Table I) showed that no CNS-unmyelinated fibres were larger than 0.8 μm. Below this size the fibre spectrum was similar to that of the PNS part of the rootlets. In the fibres analysed at 10 μm intervals, fibre size remained roughly the same on the two sides of the PNS-CNS borderline (Table II).

An unusual observation made in the mantle zone was the occurrence of round myelinated profiles about 5 μm in diameter They contained swollen mitochondria, round membrane demarcated dense bodies 0.1-0.2 μm in diameter and vesicular entities (Fig. 10b). The surrounding myelin sheath consisted of 2 10 lamellae. Serial section analysis showed that these myelinated profiles measured 5-10 μm in length and diminished in size to 0.5-1 μm in the distal direction where they showed all the characteristics of a small myelinted or unmyelinated axon (Fig 10a). When traced proximally the profiles were found to taper off (Fig 10c) and finally to disappear completely (Fig 10d). The profiles did not send out side branches.

Unmyelinated fibres at the passage of the PNS-CNS borderline

The majority of the unmyelinated fibres crossed the PNS-CNS borderline in the proximal part of the transitional region, then aggregating in the ventrolateral aspect of the rootlet (cf. Berthold and Carlstedt, 1977). A minority of the fibres entered the CNS compartment more distally in the TR. Such fibres either remained in the central parts of the core zone or left the CNS compartment only to re-enter at a more proximal level (Figs. 4 and 6) (see below).

Unmyelinated fibres in the transitional region

Three different modes of C-fibre PNS-CNS transition were observed. The mode of transition appeared to be related to the diameter of the axons.

1) Fibres less than 0.6 µm in diameter These axons continued as unmyelinated into the CNS compartment. At the point where a bundle of C-fibres was about to cross the PNS-CNS borderline the Schwann cell cytoplasm surrounding the individual axons attenuated and terminated (Figs. 3 a-d). At the transition some fibres were covered partly by strands of Schwann cell cytoplasm and partly by astrocyte processes (Figs. 3b c). Other fibres lost their investing Schwann cell coat a few µm distally to the PNS-CNS borderline and were closely apposed to its neighbour axons in the bundle (Fig 3c) or ran solitarily being covered only by a basement membrane

At the point of transition several unmyelinated fibres revealed, when cross-cut, a picture similar to that of a cross-cut small node of Ranvier that is the axon was more or less surrounded by radially arranged delicate »finger-like» cytoplasmic processes 500-1000 Å thick (Figs. 3b 4b c, d, 5 9b) These processes were of astrocytic origin and reached to within less than 100 Å from the axolemma. Between the tip of a process and the axon membrane there were accumulations of moderately electron dense material. At these sites, on the axoplasmic side of the axolemma, there was material of a high electron density (Figs. 5 9b). In contrast to a genuine node of Ranvier the occurrence of processes was restricted to certain parts of the axonal perimetry Examination of series of consecutive cross and longitudinal sections showed that the astrocytic processes in fact were thin »leaflets-like» extensions which ran along the axon for distances up to 20µm (Fig 7)

The arrangements given above were prevalent and more elaborated outside larger axons (> 0 4 µm) than outside smaller ones The latter were as a rule surrounded by some blunt astrocyte processes lacking membrane specializations (Fig 3c). Larger axons (0.4 µm) were at the transitions often separated from their neighbouring axons by astrocyte extensions (Fig 5) and were closely spaced with other axons a few µm more proximally

2) Fibres 0.8 µm or more in diameter These fibres which constituted some 5% of all unmyelinated axons in the PNS compartment, could not be recovered as unmyelinated on the CNS side of the boderline Instead, careful serial section analysis showed that they became myelinated as they crossed the boderline and entered the CNS (Figs 7 and 9a-c) Their myelin sheaths consisted of up to 10 lamellae. The mean myelin period was 100 Å (SD 10 Å, n 35) (cf Hildebrand, 1972). The internodal length ranged between 10 and 40 µm (Fig 7). In the approximately 5 µm gap between the PNS Schwann cell covering and the CNS myelin sheath there were astrocytic leaflets arranged as described above in section 1 (Fig 7)

The nodes of Ranvier situated more proximally along these fibres (Fig 7) showed a rather simple architecture with broad node-gaps containing occasional astrocyte extensions i e. they appeared quite like the commonly described small CNS node of Ranvier in the central nervous system (cf Bunge 1968).

3) Fibres some 0 6-0.8 µm in diameter Most of these fibres showed segmental myelination of their CNS part. Here short myelin sheath cuffs 3-20 µm long were intercalated in an irregular manner between unmyelinated stretches (Fig 8) Some fibres showed the arrangements of axons 0.8 µm or more in diameter at both ends of the myelin investment.

DISCUSSION

Redistribution of C-fibres. In full agreement with earlier observations (e.g. Gasser 1955; Peyronnard *et al.*, 1973; Aguayo *et al.* 1976) the Schwann cell internuclear distance was about 100 μm and no major shifts were noted in the grouping and distribution of C-fibres at their passage along the root halfway between the spinal ganglion and the spinal cord. The passage along the TR, on the other hand, meant that the C-fibres deserted the central parts of the rootlet and became redistributed to its outer layers and in particular to its ventrolateral aspect (Berthold and Carlstedt, 1977). This is illustrated in the present work by the high degree of C-fibre divergence (or criss-crossing) as noted in the glia fringe close to the PNS-CNS borderline, where the average Schwann cell internuclear distance measured about 12 μm. The occurrence of a Schwann cell inter-nuclear distance that is some 0.1 time that of more distal root regions indicate that there are more Schwann cells per unit length unmyelinated axon in the TR than in the stereotype part of the root. It was found that the average number of unmyelinated fibres connected to a Schwann cell perikaryon was about twice as high in the stereotype part of the root than in the TR. Provided that those observations, derived from a small number of observations (n=30) are valid, there should be at least 20 times as many Schwann cells per unit length unmyelinated axon in the TR as compared to the stereotype part of the root. Such an unusually high occurrence of C-fibre-related Schwann cells might be an effect of the excessive C-fibre criss-crossing or it might reflect a genuine Schwann cell surplus of obscure origin specific for the TR.

Whether the fibre redistribution implies a regrouping of unmyelinated axons at random or whether the axons become segregated according to some morphological and functional principle is unknown. Nevertheless it should be mentioned that the CNS arrangement of the redistributed unmyelinated axons — close-packing of many fibres without intervening glia might, in pathological conditions, allow interaction. This matter has recently been discussed by Sunderland (1976) as a phenomenon that might be involved in *causalgia*

C fibres at the CNS entrance: PNS calibre spectra show that 80-90% of the unmyelinated axons measured 0.4-0.7 μm (Table I). About 5% of the axons measured 0.8 μm or more (upper limit 1.2 μm). CNS spectra show a similar distribution except for the remarkable lack of axons more than 0.7-0.8 μm thick. Serial section analysis showed that the disappearance of large unmyelinated axons on the proximal side of the PNS-CNS borderline was due to the fact that axons 0.8 μm or more in diameter acquired a complete myelin sheath as they entered the CNS compartment. This explains the already reported (Berthold and Carlstedt, 1977) surplus in small myelinated fibres as noted proximally in the TR and also excludes the possibility that discrepancies in the occurrence of unmyelinated and small myelinated fibres at different levels in the TR to any appreciable extent depended on C-fibre termination in combination with branching of small myelinated fibres.

Serial section reconstructions, however revealed that about 1 ‰ of the C-fibres did in fact terminate in the CNS compartment close to the borderline (Figs. 10a-d). These axons showed bulbiform terminal swelling with an appearance similar to that observed by Lampert (1967) after transection of the dorsal columns in rats. Thus signs of C-fibre degeneration appear to be part of the normal TR morphology.

A fully myelinated condition of the large C-fibres was followed in the CNS up to a distance of 100 μm proximally to the PNS-CNS borderline. Whether these fibres remained myelinated in the CNS proper is unknown. However since these fibres in analogy with afferent fibres in general should divide into a cranial and a caudal branch and since branching means a decreased fibre size (Lehmann, 1959) it is not unlikely that C-fibre myelination might be a phenomenon peculiar to that fibre

segment which extends between the PNS-CNS borderline and the first branching point in the CNS. The same arguments apply to the segmentally myelinated C-fribres measuring 0.6-0.8 μm in diameter. Several internodes of the latter fibres were less than 10 μm in length, a size comparable to that of the very short internodes as described by Waxman (1970) in the teleost brain. These observations may be of some interest with regard to the debate on the signal for myelination (see, for instance, Speidel 1964, Matthews 1968, Friede 1972; Weinberg and Spencer 1975). They show that this signal, whatever it might be, gives rise to a situation which appears to be strikingly dependent on axon size: feline unmyelinated PNS axons (upper size limit 1.5 μm) acquired a more or less complete myelin sheath in the CNS if they measured more than 0.6 μm.

The functional significance of myelin investment of thin axons has been much discussed. Rushton (1951) calculated that axons less than 1 μm in diameter would transmit impulses faster if unmyelinated. It has been suggested that the increase in conduction velocity that takes place during postnatal development is not directly related to myelination (Conway *et al.*, 1969). Koles and Rasminsky (1972) computed the theoretical effects of demyelination on the conduction of impulses and found that even a few myelin lamellae are of importance for conduction properties. Stone and Freeman (1971) found an eightfold increase in conduction velocity between the intraretinal (unmyelinated) and extraretinal (myelinated) part of even the slowest conducting fibres, which in the intraretinal position presumably had a diameter less than 1 μm (see also Stone and Holländer, 1971). According to the theoretical calculations by Waxman and Benett (1972) conduction velocity shall accelerate when an unmyelinated fibre is myelinated: the conduction velocity was calculated to be approximately 2.6 times larger in an myelinated fibre, 1 μm diameter, as compared to a non-myelinated 1 μm axon (Waxman, 1975).

Against this background it can be tentatively suggested that the mode of impulse conduction changes and that conduction velocity increases in large C-fibres as they enter the CNS compartment of the TR. According to the meagre data relating receptive properties to C-fibre size, the large (> 1 μm) axons should transmit impulses set up in low-threshold mechanoreceptors (Douglas and Ritchie, 1957, Besson and Perl, 1969).

Whether the relationships noted between astrocytes and the bordeline segments of C-fibres 0.4-0.7 μm in size i.e. node-like arrangements (see Fig. 9) are functionally significant for impulse-propagation is unknown. However, the ultrastructural organization noted at the transition of medium and large C-fibres become particularly interesting in view of Waxman's (1975) ideas on the correlation between local nerve fibre design and function. Thus Waxman discusses the possibility that the integrative properties of impulse transmission is influenced not only by the nerve cell body, the dendrites and the receptors but also by local arrangements of the interconnecting fibre.

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Table I

Fibre spectrum of unmyelinated fibres on the peripheral (P) and central (C) side of the PNS-CNS borderline

Specimen	–0.35 µm	0.35 – 0.46 µm	0.46 – 0.57 µm	0.57 – 0.68 µm	0.68 – 0.79 µm	0.79 – 0.90 µm	0.90 – 1.01 µm	1.01 – 1.12 µm	1.12 – 1.23 µm	1.23 – 1.34 µm	No. of fibres
K 1028 P	33	131	133	33	8	9	2	1			337
C	38	148	123	15							324
K 1038 P	47	198	344	287	75	10	8	3	2		980
C	39	254	300	236	35						864
F 1040 P	46	330	351	210	64	11	5	2	1		1010
C	43	277	386	239	34						989

Table II

Axon size as found on the two sides of the PNS CNS borderline in unmyelinated and unmyelinated myelinated axons.

		PNS					Borderline	CNS				
Specimen	Number of axon	50 µm	40 µm	30 µm	20 µm	10 µm	0	10 µm	20 µm	30 µm	40 µm	50 µm
	1	[illegible]	[illegible]	[illegible]	0.86	0.86		0.7^{m}	[illegible]	1.07^{m}	1.07^{m}	1.29^{m}
	2	0.41	[illegible]	0.41	[illegible]	0.30		0.41	0.41	0.41	0.41	0.30
	3	0.41	0.41	0.41	0.41	0.30		0.41	0.41	0.41	[illegible]	0.41
	4	0.63	0.63	0.63	[illegible]	0.74		0.63	[illegible]	[illegible]	0.63	0.63
K 1028	5	0.41	0.41	0.41	[illegible]	0.30		0.30	0.41	[illegible]	0.41	0.30
	6	[illegible]	0.41	0.41	0.30	0.41		[illegible]	0.41	[illegible]	[illegible]	[illegible]
	7	0.74	0.74	0.74	0.74	0.74		0.74	0.63	0.63	0.74^{m}	0.74^{m}
	8	[illegible]	[illegible]	[illegible]	[illegible]	0.4		[illegible]	[illegible]	0.41	0.41	0.41
	9	0.4	[illegible]	[illegible]	0.41	0.30		0.41	[illegible]	[illegible]	[illegible]	[illegible]
	1	0.63	[illegible]	[illegible]	0.63	0.63		[illegible]	0.74^{m}	0.74^{m}	0.74^{m}	0.74^{m}
	2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		0.41	0.41	0.4	0.41	0.30
	3	0.4	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
		0.63	0.63	0.63	[illegible]	[illegible]		[illegible]	0.74^{m}	[illegible]	[illegible]	0.74^{m}
K 1038	5	[illegible]	0.41	0.41	0.41	0.41		[illegible]	[illegible]	[illegible]	[illegible]	0.41
	6	0.30	0.41	0.41	0.41	0.41		[illegible]	[illegible]	0.41	[illegible]	[illegible]
	7	0.63	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]	[illegible]	[illegible]	0.74^{m}	0.74^{m}
	8	[illegible]	0.41	0.41	0.41	[illegible]		[illegible]	[illegible]	[illegible]	0.63	[illegible]
	9	[illegible]	0.41	0.41	0.41	0.41		0.41	[illegible]	0.41	0.41	0.41
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]	1.1^{m}	[illegible]	1.07^{m}	1.07^{m}
	2	0.41	[illegible]	[illegible]	[illegible]	0.4		0.30	[illegible]	0.41	0.41	0.41
	3	0.41	[illegible]	0.41	0.30	0.30		0.41	0.41	0.41	0.41	0.30
	4	0.63	0.63	0.63	0.63	[illegible]		0.41	[illegible]	[illegible]	0.74^{m}	0.74^{m}
040	5	[illegible]	0.41	0.41	0.4	0.41		[illegible]	[illegible]	0.41	[illegible]	0.30
	6	0.30	0.30	0.30	[illegible]	0.30		0.41	0.41	0.41	0.41	0.41
	7	[illegible]	[illegible]	[illegible]	0.41	[illegible]		[illegible]	[illegible]	[illegible]	[illegible]	0.41
	8	0.63	0.63	0.41	[illegible]	[illegible]		0.41	[illegible]	0.30	[illegible]	[illegible]
	9	0.63	[illegible]	[illegible]	[illegible]	0.41		[illegible]	[illegible]	0.30	[illegible]	[illegible]

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LEGENDS TO FIGURES

Abbreviations used in figures and legends.

Ap	astrocyte process
Ax	axon
BM	basement membrane
C	collagen fibres
G	gliosome
My	myelin
M	mantle zone
R	node of Ranvier
S	Schwann cell cytoplasm

All pictures beside Fig 2 are lectronmicrographs.

Fig 1a Cross-section. Bundle of PNS unmyelinated axons. Most axons are separated by Schwann cell septa (x33000)

Fig. 1b Cross-section. Bundle f CNS unmyelinated axons. The axons are closely packed and accompanied by astrocyte processes (Ap) and small myelinated fibres (x37000).

Fig 2 Diagrammatic representation f Schwann cell nuclear length and the distribution of Schwann cells along bundles of unmyelinated fibres in the transitional region. Three bundles each of three specimen (K1026, K1039 K1040) were used.

Figs 3a-d Four different cross levels selected from complete series of consecutive cross-sections through the transitional region. The section number is given in the lower left corner of each picture. Section thickness is about 700 Å. A bundle of 12 axons is followed from the PNS to the CNS compartment in the proximal part of the TR. Each fibre is indicated by a number The mantle zone (MZ) of the CNS compartment is observed in the lower part of the tures. Cross cut collagen fibres (C) are seen in the endoneural space. The axons are surrounded by Schwann cell cytoplasm (S) distal to the PNS-CNS borderline (Fig. 3a). At the borderline (Fig 3b) some 26 μm proximal to the level represented in Fig. 3a, fibre 1 is partly invested by slender Schwann cell septa (S) and partly invested by broad irregularly shaped or slender radially arranged finger-like astrocyte processes (Ap). At the place of transition from the PNS to the CNS (Fig. 3c) some 5μm proximal to the level represented in Fig. 3b, fibres 1 2, 4 5, 7 forms a bundle of closely packed axons. The bundle is surrounded partly by slender processes of Schwann cell cytoplasm (S) and partly by delicate astrocyte processes of the mantle zone (MZ) Proximal to the PNS-CNS borderline (Fig 3d) and some 46 μm from the level represented in Fig 3a, the axons are closely packed in a bundle surrounded by the astrocyte processes of the mantle zone (MZ) (x22000).

Fig 4a-f Six different cross levels selected from a complete series of consecutive cross-section through the TR Section number is given in the lower left corner of each picture. Section thickness is 700 Å. Two unmyelinated fibres cross the PNS-CNS borderline two times in the distal part of the TR. The fibres are invested by Schwann cell cytoplasm (S) distal to the PNS-CNS borderline (Fig 4a). At the borderline (Fig. 4b) the basement membrane (BM) of the Schwann cell and th astrocytes of the mantle zone are continuous. Schwann cell (S) and astrocyte derivatives are pposed without intervening basement membrane (Figs. 4b, d,). Multiple slender astrocyte lamellae derived from gliosome (G) containing astrocyte processes are directed towards the fibres (Fig. 4b, c, d) Th fibres are situated in the CNS compartment for some 18 μm (Figs 4b c, d) In Fig. 4f some 22 μm proximal to the level represented in Fig. 4a the fibres are again invested by Schwann cell cytoplasm (S) in the PNS compartment (x29000).

Fig. 5 Cross-section A bundle of unmyelinated fibres invested by Schwann cell cytoplasm (S) is seen in the upper part of the picture. In the centre of the picture an unmyelinated axon is situated at the PNS-CNS borderline. The fibre is partly surrounded by a basement membrane (BM) and radially arranged delicate finger-like processes some 500-1000 Å thick, derived from gliosome (G) and fibrillae containing astrocytic extensions. There is a material of high electron density on the axoplasmic side of the axolemma where the astrocyte processes points towards th axon (arrows) (x39000).

Fig 6 Two cross sectioned unmyelinated axons (Ax) in a process of the glial fringe in the distal part of the transitional region. The axons are surrounded by numerous irregularly shaped, gliosome (G) and fibrillae containing astrocytic extensions (x38000).

Fig. 7 Photomontage. Longitudinal section through a 0.8 μm thick nerve fibre crossing the PNC-CNS borderline (hatched line) In the lower part of the picture the fibre is unmyelinated and invested by Schwann cell cytoplasm (S). In its most distal CNS part, the unmyelinated fibre is invested by lamellae like astrocytic processes (Ap). Some 4 μm proximal to the PNS CNS borderline the axon is myelinated (My) and thus a small CNS myelinated fibre. The most distally situated node of Ranvier (R) is seen in the upper part of the picture. Occasional glial processes are seen in the broad node gap. The internodal length is 10 μm and the myelin is seen to terminate at both internodal ends in a conventional manner (x11000).

Fig. 8 Photomontage. Longitudinal section through a segmentally myelinated axon (Ax) in the mantl zone of the transitional region The fibre is about 0.6 μm thick. The internode in the lower part of the picture is about 6 μm long. The fibre is invested by astrocytic processes (Ap) in its unmyelinated part (x11000).

Figs, 9 a, b, Three levels from a complete cross section series through the TR, showing a nerve fibre, 0 9 μm thick passing the PNS-CNS borderline.

) The fibre is unmyelinated distal to the borderline and surrounded by Schwann cell cytoplasm (S). The endoneural space surrounding the fibre is demarcated by astrocytic processes of the mantle zone. Some 3 μm more proximally (Fig 9b), the fibre is invested by broad irregularly shaped and slender radially arranged, leaflet-like astrocytic processes (Ap). There is an electron dense material subjacent to the axon membrane at those places where the aleaflet-like astrocytic processes are directed towards the axon (arrows). Some 10 μm proximal to the level shown in Fig 9b the axon is myelinated (Fig 9c) (x24000).

Figs. 10 a-d Series of cross-section. A small myelinated CNS fibre (x) (Fig, 10a) tranforms proximally into an enlarged myelinated profile containing mitochondria, dense bodies and vesicular entities (Fig. 10b) About 6 μm proximal to the level shown in Fig. 10b the fibre has tapered off into a tip (Fig 10c)and some 2 μm more proximally the fibre has disappeared completely (x19000).

Figures at the end of this volume

Running title Unmyelinated fibres in the transitional region

Unmyelinated fibres in the transitional region

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Observations on the Morphology at the Transition between the Peripheral and the Central Nervous System in the Cat. V

A light microscopical and histochemical study of S_1 dorsal rootlets in developing kittens

by

C.H BERTHOLD and T CARLSTEDT

ABSTRACT

C. H. BERTHOLD and T CARLSTEDT *Observations on the morphology at the transition between the peripheral and the central nervous system in the cat. V A light microscopical and histochemical study of S dorsal rootlets in developing kittens.* Acta physiol. scand. 1977 Suppl. 446 73-85.

The postnatal development of the transitional region (TR) i.e. the proximal free part of a spinal rootlet that contains both PNS and CNS tissue, was studied light-microscopically in semi-thin sections and after histochemical staining according to the Marchi and OTAN methods for the demonstration of degenerating myelin and ccording to the Gomori method for the demonstration of acid phosphatase activity In the newborn kitten the PNS tissue extended well up to the spinal cord surface and the rootlets lacked transitional region. The CNS tissue entered the root during the second postnatal week, and transitional region was fully established t the beginning of the second month.

The degree of myelination in the group of large fibres differed on the two sides of the PNS-CNS borderline: well myelinated PNS fibres were transformed into poorly myelinated or pparently unmyelinated CNS-fibres. PNS and CNS myelin sheaths of large fibres ppeared to be of equal thickness in the 4 week old kitten.

During the first postnatal month large amounts of Marchi positive material and a high acid phosphatase activity occurred in complex paranodes and very short internodes in the PNS compartment just distally to the PNS CNS borderline In the adult cat Marchi positive bodies were numerous in the CNS compartment just proximally to the PNS-CNS borderline.

The results are discussed against previous studies on focal demyelination as found during the normal development of the feline peripheral nervous system.

The systematic investigation of the anatomical correlate of postnatal spinal reflex maturation in the hindlimbs of the cat as initiated by Skoglund more than 15 years ago (Skoglund, 1960a, b c, d, e, 1966) includes by now treatises on peripheral nerve fibre maturation (Skoglund and Romero 1965 Ekholm, 1967 Schwieler 1967 Berthold, 1968, Nyström, 1968), motor neuron maturation (Conradi 1969; Mellström, 1971, Hammarberg, 1976; Ronnevi, 1976) central nerve fibre maturation (Hildebrand, 1971) and skeletal muscle maturation (Nyström, 1968; Hammarberg 1976) Common to all these studies is the demonstration of a profound and rapid maturation which includes steps of remodelling and degeneration and which takes place during the first postnatal month. This development is well functional improvements and gives fully or almost fully mature

Development of the transitional region

patterns during the 5th-6th postnatal week. So far no studies at all have been devoted to the postnatal development of the feline transitional region (TR) i.e. that part of the nervous system where the neuronal environment changes from the CNS to the PNS pattern. The only available data on the developing TR seems to be that given by Henschen (1915) in a study on acoustic nerve tumors where he comments that the extent of CNS tissue into the eighth cranial nerve of the infant is considerably shorter than that of the adult.

Against this background and taking into account the fact that the mature feline transitional region ultrastructure has recently for the first time been described (Carlstedt, 1977 a, b; Berthold and Carlstedt, 1977 a, b) the undertaking of a survey study of the developing transitional region becomes more or less a matter of course.

The present communication deals with the development of this region as studied light-microscopically in semi-thin sections of plastic-embedded specimens and reports the outcome of two histochemical tests on the occurrence of myelin degeneration products and acid phosphatase

The results, which strongly emphasize the need of further ultrastructural analysis of the postnatally developing transitional region are mainly discussed in terms of the focal demyelination previously demonstrated in the peripheral nervous system of the kitten (Berthold and Skoglund, 1968, Berthold, 1973c).

MATERIAL AND METHODS

In all, 5 adult cats and 31 kittens (0-180 days old) were used. All animals were anaesthetized intraperitoneally with pentobarbital (40 mg/kg body weight). The lower lumbar and sacral spinal cord segments and their spinal roots were fixed by vascular perfusion through the thoracic aorta (Berthold, 1968a)

That preparative procedure which previously has been given for the TR (Carlstedt, 1977a) was used for adult cats and kittens more than 2 months of age. In younger kittens (see Table I) the perfusate consisted of 5% glutaraldehyde dissolved in 300 mOsm Millonig buffer containing 2.7% low-molecular dextran (Berthold, 1968a) After perfusion fixation and a laminectomy the S_1 dorsal spinal roots and their spinal cord segment were removed. The roots were carefully separated into the individual rootlets with the aid of watchmakers' forceps. After trimming with a razor blade the specimens consisted of the proximal one third of the rootlet and its attachment to the spinal cord together with a small piece of the surrounding white matter The specimens were placed for 4 hours at +4°C in a glutaraldehyde solution of the same composition as the perfusate and were rinsed overnight in th buffer at 4°C Three rootlets per animal were used for Marchi and OTAN treatment (see below). Remaining specimens were osmicated in 2% OsO_4 in 300 mOsm Millonig buffer for four hours, dehydrated in graded series of acetone solutions and embedded in Vestopal W (Ryter and Kellenberger, 1958). Longitudinal semi-thin sections (0.5 µm thick) were cut at random on a LKB 4800 Ultrotome, placed on glass slides and stained with alkaline toluidine blue (Carlstedt, 1977a)

The Marchi and OTAN methods (see Adams 1965) Longitudinal 15-30 µm thick sections obtained on an Oxford Vibratome's were immersed overnight (18-20 hours) in a mixture of 1/4 1% OsO in distilled water and 3/4 1% $KClO_3$ in distilled water (i.e. the Marchi medium, cf. Adams, 1965). In order to obtain OTAN-staining Marchi-stained slices were rinsed for a short time in a phosphate buffer and treated in a prewarmed saturated solution of alpha-naphthylamine at 37°C for 5-10 minutes. OTAN staining was performed only in kittens.

Some sections were investigated directly after glutaraldehyde fixation or after additional treatment in 0.25% OsO in distilled water

The Gomori method (Gomori, 1952) In one cat and 6 kittens (0, 7 14, 21 30 and 45 days old) the perfusate consisted of 2.5% glutaraldehyde dissolved in a 0.1 M cacodylate buffer adjusted to 300 mOsm by the addition of NaCl and containing 2.7% low-molecular dextran. The glutaraldehyde stock solution was purified by shaking with active charcoal (Fahimi and Drochmanns, 1965). The perfusion fixation, which duration varied from 10 to 20 minutes, was concluded by a rinse with the buffer lacking glutaraldehyde After removal the specimens were sectioned into longitudinal slices (15-30 μm thick) on an Oxford Vibratome[1] The slices were incubated in a freshly prepared Gomori medium (Gomori, 1952) with sodium-ß-glycerophosphate as substrate for 15 to 30 minutes at 37°C. After a rinse in th cacodylate buffer the specimens were »developed» for 30 seconds in 1% ammonium sulphide and mounted in glycerine on glass slides. Control specimens were either treated with 1% NaF before incubation or were incubated in a medium which lacked substrate.

RESULTS

Toluidine-blue-stained semi thin sections

After toluidine-blue staining all Vestopal-embedded specimens revealed PNS myelin sheaths with fairly smooth internodal contours and of an intense bluish-black colour CNS-myelin sheaths, on the other hand, showed corrugated contours and appeared with a more pale blue colour These differences were used for the indentification of the PNS-CNS borderline (Figs. 1 7). A picture of the longitudinally cut mature TR and the adopted nomenclature is given in Fig 1 and its legend (cf. Berthold and Carlstedt, 1977a).

In kittens less than a week of age (Figs. 2-5) the proximal ends of S rootlets were completely free of CNS tissue (Fig. 2) PNS-myelinated fibres extended proximally to the very level of the rootlet-cord junction. PNS myelin sheaths were consistently much thicker than CNS sheaths as seen in the same fibres on the two sides of the PNS-CNS borderline. Numerous unmyelinated axon segments measuring up to 50 μm in length were found intercalated between myelinated PNS and CNS internodes at the borderline The axons of several large PNS-myelinated fibres (2 3 μm in diameter) appeared to continue unmyelinated in the CNS (Fig 3). The occurrence of cell bodies was high on both sides of the borderline: Schwann cells and immature glia cells dominated the picture in the PNS and the CNS respectively Several mitoses were seen among the glia cells. With the exception of Schwann cells connected to myelinated axons light microscopy did not allow a classification of the various glial and endoneural cell elements.

At the end of the 1st postnatal week, PNS borderline paranodes, showed a moderate dilatation, a highly tortous myelin sheath contour and a content of myelinoid bodies, i.e. the characteristics of a complex paranode (see Berthold, 1973c) To this added the occurrence of a few typical short and very short internodes 50-150 and 10-50 μm in length, respectively (Fig. 5).

During the 2nd and 3rd postnatal weeks CNS-tissue entered and occupied an expanding compartment of the proximal segment of the rootlets (Fig 4). At the end of the period the CNS compartment measured up to 50 μm in length and the rootlets displayed a definite transitional region (Fig. 6). The 2nd postnatal week brought a dramatic increase in the occurrence of very short internodes (Fig 5). The latter together with unclassified endoneural cells and complex paranodes, contained clusters of myelinoid bodies. Unmyelinated borderline axon segments were rare at the end of the 3rd week. The clear-cut difference between PNS and CNS myelin sheath thickness which was one of the main characteristics of the borderline during the first postnatal fortnight became less pronounced (Fig. 7).

During the subsequent month (postnatal weeks 4 to 8) the CNS compartment increased gradually in length (Fig 6) and measured well above 100 μm in several rootlets in the 2 month-old kitten

A close to mature overall picture seemed to be present already in 4-5-week-old animals At the beginning of this period, however it was common to find thin bundles of only a few CNS fibres accompanied by some astrocytes. Such rays of CNS tissue projected further distally than the main part of the CNS compartment (Fig 7)

Marchi and OTAN staining

In the adult cat Marchi treatment gave pale yellowish-grey myelin sheaths (Figs.9-11) The PNS-CNS borderline was characterized by the high number of borderline nodes and the less well preserved condition of the adjoining CNS myelin sheaths (cf. Carlstedt, 1977a) In the CNS compartment there were numerous black Marchi-positive bodies 2 10 μm in size (Fig 9). The bodies were concentrated in the mantle zone and were less abundant in the core zone. Brownish-black highly refractile Marchi positive granules less than 1 μm in size, were scarce in the CNS compartment. In the PNS compartment solitary Marchi positive bodies and short rows of Marchi positive granules were confined to the Schwann cell cytoplasm of a few paranodes.

Material reminiscent of Marchi positive bodies and granules were not found in specimens which after the glutaraldehyde fixation were immersed in 0.25% OsO_4 (Fig 8) Examination of specimens treated only with glutaraldehyde revealed the occurrence, particularly in the mantle zone, of round bodies with a texture similar to that of the myelin sheath (cf. Fig 16) These bodies measured 2 10 μm in size and will be referred to as myelinoid bodies (cf. Berthold and Skoglund, 1968, Hildebrand, 1971).

During the first postnatal month Marchi-positive bodies and Marchi-positive granules were observed in large numbers just distally to the PNS-CNS borderline (Table I Fig 12). After OTAN staining the blackness of these bodies and granules became more intense and the myelin sheaths appeared redbrown and much more distinct than after staining in the Marchi medium alone. In the first week the Marchi positivity consisted mainly of Marchi positive granules. The highest occurrence of Marchi positivity was noted in the 2nd and 3rd postnatal weeks, then consisting mainly of Marchi positive bodies. From the 4th week onwards the Marchi-positivity of the PNS compartment declined and the mature picture appeared at the beginning of the second postnatal month (Table I)

The Marchi positive bodies were found in complex paranodes (Fig 13) in very short internodes (Fig 14) and in unclassified endoneural cells. A comparison between the more distal parts of a rootlet and the PNS compartment of its transitional region showed that the occurrence of Marchi-positive very short internodes at the latter site indeed was remarkably high.

In the CNS compartment Marchi positive material was scarce during the first postnatal weeks (Figs, 12, 13). It occurred mainly in the form of Marchi positive granules. Marchi positive bodies began to accumulate at the end of the first month. They then became increasingly more numerous and the adult pattern was present at the end of the second month (Table I)

Gomori staining

In the adult cat staining according to the Gomori method (Figs. 15, 16) gave rise to a finely granular dark brown precipitate (reaction product) occurring on a yellowish background.

Ring and crescent-shaped accumulations of the reaction product were related to some myelinoid bodies (Fig 16) Schwann cell perikarya and the mantle zone contained a few scattered minute reaction product granules.

The precipitates referred to above were not present in control incubations. As noted elsewhere (Berthold, 1973b) control incubations showed the occurrence of a false reaction product at the nodes of Ranvier (see Fig. 15).

Throughout the postnatal period the occurrence of acid-phosphatase positivity was higher in the PNS compartment than in the CNS compartment (Figs. 17 19). The occurrence of activity was at its peak from the end of the 1st postnatal week until the beginning of the 4th week, whereafter it declined, reaching the adult level at about the end of the second month.

In newborn kittens (0-4 days of age) the reaction product appeared as 0.5-2 μm large solitary brown-black granules or bodies (Fig. 17). Most cells found close to the PNS-CNS borderline contained a few scattered granules of this kind. Accumulations of reaction products forming rings and crescents were rare.

During the 2nd and 3rd postnatal weeks the occurrence of solitary Gomori-positive granules decreased. Instead there was a high occurrence of ring- and crescent-shaped brown-black material surrounding translucent globules 2-5 μm in size. These characteristic profiles of reaction products were often arranged in clusters or rows situated in the complex paranodes and in very short internodes (Figs. 18, 19).

In the CNS compartment, where the occurrence of acid phosphatase activity remained low during the whole maturation process, the reaction product occurred in the glia cells and consisted of tiny granules and globules.

DISCUSSION

The present results have given the principal features of the postnatal development of the feline transitional region as studied light-microscopically in semi-thin sections and after histochemical incubations according to the Marchi and OTAN methods for the demonstration of degenerating myelin and to Gomori s method for the demonstration of acid phosphatase activity. Methodological aspects on these procedures as applied to PNS and CNS nerve-fibres have been discussed at length elsewhere (Hildebrand and Skoglund, 1971b Berthold, 1973a). Briefly it has been shown that Marchi-positive bodies occurring in the paranodal regions of Schwann cell cytoplasm and in relation to the outside of the paranodal myelin sheath of CNS fibres ultrastructurally correspond to lamellar myelinoid bodies. The Marchi-positive granules on the other hand are claimed to represent lipid droplets. Further, that light-microscopically observed clumps rings and crescents of acid phosphatase activity reflect the presence of aggregations of an electron-dense dust like material — the reaction product — in relation to the myelinoid bodies mentioned above (Berthold, 1973b) Scattered granules of reaction product observed light-microscopically both in Schwann cells and in glial cells can be interpreted as reflecting lysosomal and Golgi cistern phosphatase activity (cf. Friede, 1966; Holtzman and Novikoff, 1967 Berthold 1973b).

In agreement with earlier observations (Berthold 1973a, b) on adult PNS-nerve fibres, only some of the paranodes of the PNS compartment of the mature TR contained Marchi-positive bodies and mere indications of acid phosphatase activity The CNS-compartment, on the other hand, was more or less outlined by Marchi positive bodies lodging in the mantle zone. (cf. Singer and Münzer 1888). The acid phosphatase activity was low The high occurrence of myelinoid bodies in the mantle zone as suggested by the high number of Marchi positive bodies is fully consistent with our earlier electron-microscopic analysis of the adult TR (Berthold and Carlstedt, 1977a). The abundance of Marchi-positive material in the

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Material reminiscent of Marchi positive bodies and granules were not found in specimens which after the glutaraldehyde fixation were immersed in 0.25% OsO_4 (Fig 8) Examination of specimens treated only with glutaraldehyde revealed the occurrence, particularly in the mantle zone, of round bodies with a texture similar to that of the myelin sheath (cf. Fig 16) These bodies measured 2 10 μm in size and will be referred to as myelinoid bodies (cf. Berthold and Skoglund, 1968 Hildebrand, 1971).

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activation or manufacture myelin exceedingly slowly during the first postnatal weeks needs electron-microscopic exploration. An ultrastructural morphometric analysis of developing TR nerve fibres also appears well worth undertaking in view of the intensifying debate on the signal for myelinations (see, e.g. Friede 1972; Weinberg and Spencer 1975).

The occurrence of unmyelinated and/or poorly myelinated fibre stretches interconnecting well myelinated parts of an axon is regardless of latent causes of particular interest in terms of impulsepropagation. A rough extrapolation of the values calculated by Koles and Rasminsky (1972) for the length of a paranodal demyelination that would induce conduction block in a large rat PNS fibre to developing large TR fibres in the cat indicates that unmyelinated stretches some 30 and 60 μm in length might block the nerve signal in the newborn and the 3-4-week old kitten respectively. Since several axons of the TR during the first month revealed apparently unmyelinated parts 50 μm or more in length the situation in the TR might turn out to be crucial for impulse propagation during the first month after birth. It should be noted that the length of the demyelinated axon segments which are at hand in the dorsal root outside the TR (Berthold, 1973c) rarely exceeds 20 μm. This should induce impulse retardation rather than block.

ad (3) Local demyelination and elimination of supernumerary internodes are claimed to be inherent features of a maturation step — the nodalization process — whereby a certain nerve fibre stretch acquires a fixed number of evenly spaced nodes of Ranvier and whereby the primitive node-paranode regions of the already myelinated axon develop their complex adult organization (Berthold, 1973c). The nodalization process, including local demyelination has been shown to be relevant for feline CNS myelinated nerve fibres (Hildebrand, 1971a) as well as for the PNS of some commonly used laboratory animals like the rabbit and the dog (Berthold, 1974). The high occurrence and the distribution pattern of short and very short internodes and of unmyelinated axons segments, the large amounts of Marchi positive material and the abundant acid phosphatase reaction product in the proximal part of the S dorsal rootlets during the first postnatal month suggest strongly that local demyelination and Schwann cell elimination are particularly prominent features of nodalization in the transitional region.

It was recently claimed (Berthold, 1973c) that local demyelination and Schwann cell elimination in developing feline lumbosacral spinal roots are the effects of internodal crowding brought about by an increase in internodal length that exceeds that of the root. Conceivably an active outgrowth of glia along already overpopulated axons might well provoke an exaggeration of the degenerative features. The extent of the Schwann cell elimination in the TR needs, however electron-microscopic evaluation.

The possibility that there might exist a pool of eliminated supernumerary Schwann cells in the transitional region — in a sense a Schwann cell hamartoma — becomes noteworthy for two reasons. First there does exist in the adult transitional region a population of small round to fusiform cells with scanty cytoplasm and of unknown origin (Berthold and Carlstedt, 1977a). The presence of a basement membrane outside some of these cells implies an epithelial origin and suggests some sort of relationship with Schwann cells. A thorough discussion regarding the classification of endoneural cells and the taxonomic significance of the basement membrane is given by for instance, Abercrombie and Johnson (1942), Nataniel and Pease (1963) and Thomas (1963 1966). Secondly, benign tumors such as the neurinomas and the Schwannomas are found in the transitional region of spinal roots and cranial nerves (cf. Henschen, 1955). Thus, for instance, the acoustic neurinoma is claimed to originate in the transitional region of the eighth cranial nerve (cf. Mincler 1972). So a straight forward demonstration of dormant Schwann cells in peripheral nerves and spinal roots would obviously be of considerable neuropathological interest.

Development of the transitional region

The occurrence of Marchi-positive material shifted during the first two postnatal months from the PNS side of the borderline to the CNS side. The main part of the PNS Marchi-positive material appeared to have developed as an effect of nodalization and in analogy with the situation in the root proper (Berthold, 1973 c) it was found to decline rapidly towards the end of the first month. Roughly at the same time CNS-paranodal Marchi positivity started to increase (cf Hildebrand and Skoglund, 1971). The close-to-adult picture was at hand in the 8-week-old animal. Thus it seems that the focal demyelinating features of nodalization of the developing TR are restricted to the PNS compartment and do not affect the CNS compartment.

ACKNOWLEDGEMENTS

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Table I

Occurrence of Marchi-positive material (Marchi-positive bodies and Marchi positive granules) in the transitional region of S_1 dorsal roots of kittens and cats

D = distal to the PNS-CNS borderline

P = proximal to the PNS-CNS borderline

Age in days	D	P
1	++	+
1	++	+
3	++	+
3	++	+
3	++	+
4	++	+
6	+++	+
7	+++	+
8	+++	+
10	+++	+
12	+++	+
14	+++	+
16	+++	+
18	+++	+
21	++	+
21	++	+
23	++	++
25	++	++
27	++	++
30	+	++
30	+	++
40	+	++
50	+	+++
60	+	+++
180	+	+++
Adult	+	+++
Adult	+	+++
Adult	+	+++
Adult	+	+++

\+ = low occurrence of Marchi-positive material
++ = moderate occurrence of Marchi-positive material
+++ high occurrence of Marchi-positive material

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LEGENDS TO FIGURES

Abbreviations used in figures and legends.

A	astrocyte
BN	borderline node
C	central nervous fibre
CNS	central nervous system
CZ	core zone
MZ	mantle zone
P	peripheral nervous fibre
PNS	peripheral nervous system
N	node of Ranvier
SC	spinal cord

All pictures are light-micrographs f longitudinal sections through th S dorsal root-spinal cord j nction. Figs. 1 7 are obtained from toluidine-blue-stained semi-thin section of Vestopal-embedded specimens. In Figs. 1, 2, 4, 6 the solid line indicates the plane of rootlet-spinal cord junction and the hatched line the PNS-CNS borderline. In Figs. 3, 5, 7 the CNS is in the upper part of the pictures.

Fig 1 Adult cat CNS tissue protudes like a cone from the dorsal rootlet-spinal cord junction into the proximal part of the rootlet. This part of the rootlet, where PNS and CNS tissue occur side by side is denoted the transitional region (TR). The CNS compartment of the TR (CNS) consists of core zone (CZ) surrounded by the mantl zone (MZ) rich in astrocytes. The nerve fibres pass from the PNS to the CNS type of organization t borderline nodes situated at the outer aspect of the mantle zone (x200)

Fig 2 Newborn kitten. PNS fibres with distinct myelin sheaths together with an abundance of cell lar profiles f various kinds are noted at the rootlet-spinal cord junction. The PNS-CNS borderline is situated at the surface of the spinal cord (x450).

Fig. 3 Same specimen as in Fig. 2. A myelinated fibre of PNS type (P) is unmyelinated in the CNS (C) (x1400).

Fig. 4 One-week-old kitten The CNS tissue bulges out into the proximal part of the rootlet. The central myelinated fibres have thinner myelin sheaths than the peripheral myelinated ones (450).

Fig 5 Same specimen in Fig. 4 A very short internode of PNS type containing a highly complex and tortous myelin sheath is situated at the PNS-CNS borderline (x1100).

Fig. 6 One-month-old kitten. CNS tissue projects like a cone from the rootlet-spinal cord junction into the proximal part of the root. The PNS and CNS fibres appear with about evenly thick myelin sheaths (450).

Fig. 7 Same specimen as in Fig. 6. A thin bundle of CNS-fibres projects into the PNS compartment. The PNS and CNS myelinated parts of the fibres meet at borderline nodes of Ranvier (arrows). An astrocyte nucleus is found in the upper left corner (x1100).

Fig. 8 Adult cat. Specimen treated with 0.25% OsO_4 after glutaraldehyde fixation. Spinal cord to the left. The dotted line indicates the PNS-CNS borderline. The CNS compartment (CNS) is stained lighter than the PNS compartment (PNS). No Marchi-positive material is observed (cf. Fig. 9) (x200).

Fig. 9 Adult cat. Marchi method. There are many Marchi positive bodies in the mantle zone and they mark out the PNS-CNS borderline (x200).

Fig. 10 Adult cat. Marchi method. Two Marchi positive bodies are situated in the central paranodal region close to the borderline nod (BN) (x1100).

Fig. 11 Adult cat. Marchi method A Marchi positive body is situated close to the borderline ode (BN) (x2200)

Fig. 12 Eight-day-old kitten. OTAN method. A high occurrence of Marchi positive bodies and Marchi positive granules is observed just distally to the PNS-CNS borderline (x1100).

Fig. 13 Ten-day-old kitten. OTAN method. Marchi positive bodies are seen in two complex paranodes distal to the borderline nodes (BN) (x1100).

Fig. 14 Twelve-day-old kitten. OTAN method A very short internode situated distally to the borderline nod (BN) is filled with Marchi positive bodies and Marchi positive granules (x1200).

Fig. 15 Adult cat. Gomori method. Two PNS paranodes at the PNS-CNS borderline contain ring and crescent like accumulations of reaction product. An accumulation of dark material (false activity) is seen in the axon of the uppermost borderline node (BN) (x1500).

Fig. 16 Adult cat. Ring and crescent-shaped accumulations of precipitate (arrows) occur outside bodies of the same texture as myelin, e.g. myelinoid bodies at the CNS paranodes proximally to th PNS-CNS borderline (x1300).

Fig. 17 Newborn kitten. There is a high occurrence of granules and bodies in the PNS compartment of the TR (x1900).

Fig. 18 Ten-day-old kitten. Ring-like accumulations of precipitate (arrows) are situated in complex paranode close to the borderline node (BN) (x1400).

Fig. 19 Twelve-day-old kitten Ring- and crescent-shaped accumulations f precipitate are present in a very short internode situated close to the borderline node (BN) (x1400).

Figures at the end of this volume

Running title: Development of the transitional region

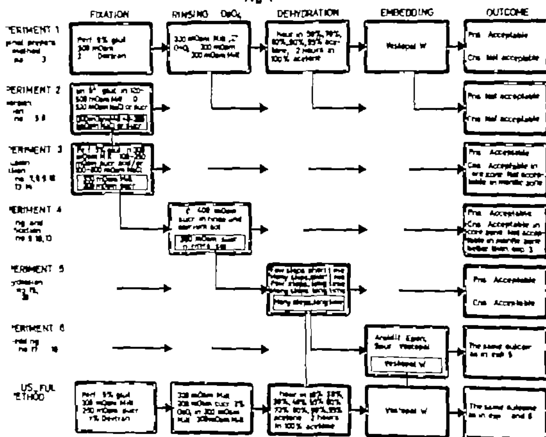
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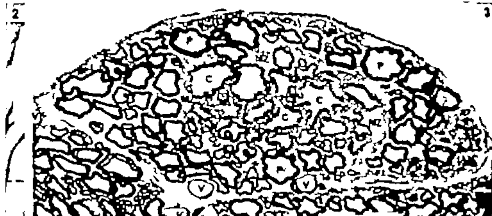
Figures to I

Observations on the morphology at the transition between the peripheral and the central nervous system in the cat. I. A preparative procedure useful for electron microscopy of the lumbosacral dorsal rootlets

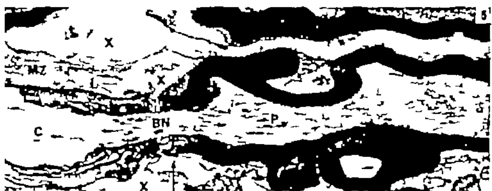
Running title EM preparation of the transitional region

Fig 1

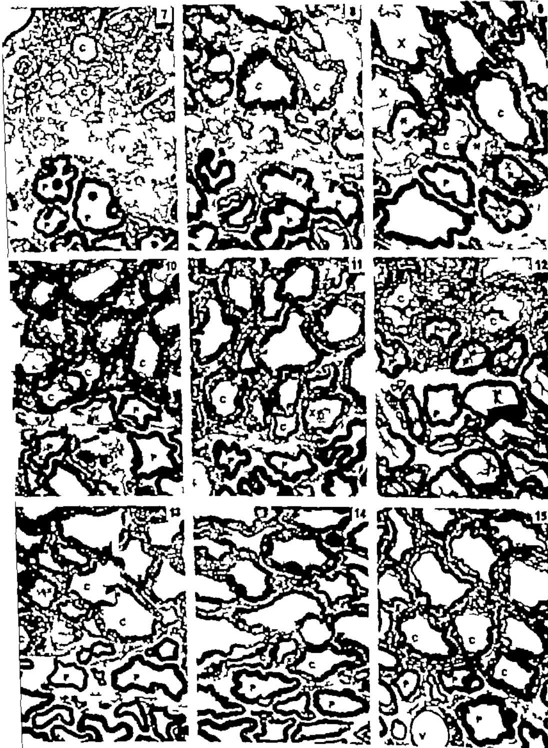

FIXATION
RINSING
DEHYDRATION
EMBEDDING
OUTCOME
Vestopal W
Pns Acceptable
Cns Not acceptable
Pns Not acceptable
Cns Not acceptable
Pns Acceptable
Pns Acceptable
Pns Acceptable
Cns Acceptable
Many steps, long time
Vestopal W
Vestopal W
100% acetone


2
3
C
C
C
MZ
V
V
V


C
P
X
MZ
P
C
P
4


X
MZ
X
C
BN
P
X
5


C
B
P
6




16
C
P
P


17
C
C
C
P
P


18
C
C
P
P


19
C
P

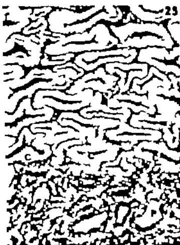

20
C
C
C
P
P


21
C
P
P
P


22
C
C
P


23
C
C
P
P
P


24
C
C
P
P
P


25
C
P


26
C
C
P
V
P
P


27
C
C
P
P


C
C
C



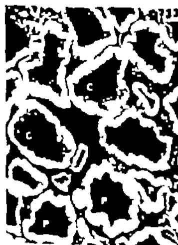

30
C
C
V
P


P
P




31
C
C
C
P
P


32
P
P

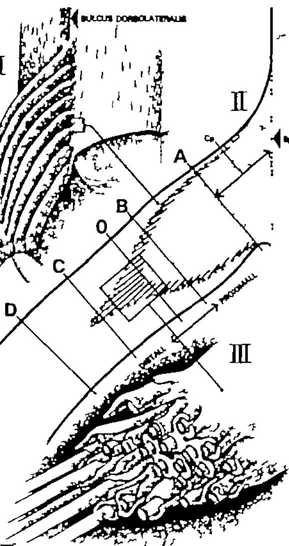

C
C
P
P

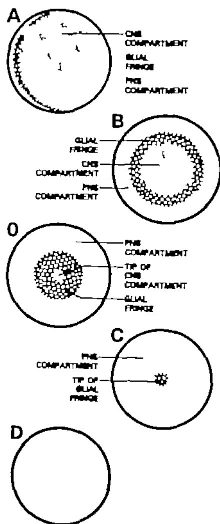
Figures to II

Observations on the morphology at the transition between the peripheral and the central nervous system in the cat. II General organization of the transitional region in S_1 dorsal rootlets

Running title General organization of the transitional region

Fig.1


SULCUS DORSOLATERALIS
I
II
III
A
B
O
C
D
PROXIMAL
DISTAL


A
CNS COMPARTMENT
GLIAL FRINGE
PNS COMPARTMENT
B
GLIAL FRINGE
CNS COMPARTMENT
PNS COMPARTMENT
O
PNS COMPARTMENT
TIP OF CNS COMPARTMENT
GLIAL FRINGE
C
PNS COMPARTMENT
TIP OF GLIAL FRINGE
D


2
SC
R
R
P
P
4a
4b


D
C
RS
D
B
A


Ve
M
D
V
L
1
6


14
MB
MB


15


16


17


BM
18a
Co

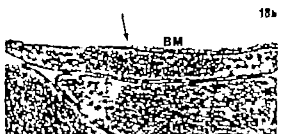

18b
BM

Fig 21

ROOTLET 1

No OF CELLS

FIBROBLAST
MICROGLIA
SCHWANN CELL MYELINATED FIBRE
UNCLASSIFIED CELL
PERICYTE

LEVEL C
LEVEL B
LEVEL

a

No OF ASTROCYTES

LEVEL C
LEVEL B
LEVEL A

b

No OF OLIGODENDROCYTES

LEVEL C
LEVEL B
LEVEL A

c

CENTRAL MYELINATED

AREA $10^3 \mu^2$

LARGE FIBRES
MEDIUM FIBRES
SMALL FIBRES
AREA OF ROOTLET
AREA OF CNS PART

LEVEL C
LEVEL B
LEVEL A

d

AREA UN
640 μ^2

AREA

AREA

e

D

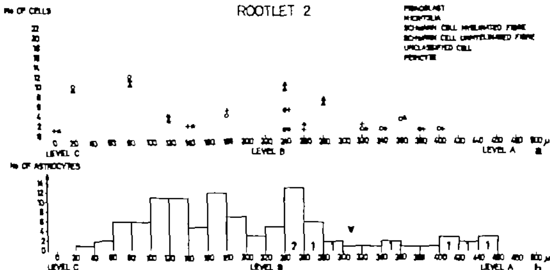
C

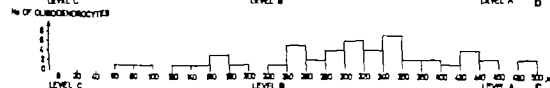
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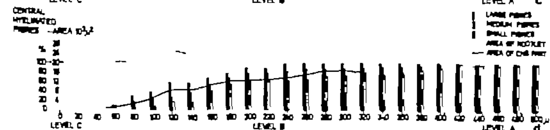
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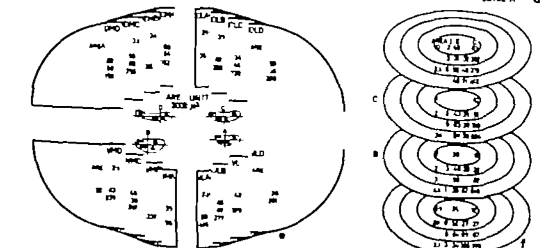
Fig 22

ROOTLET 2









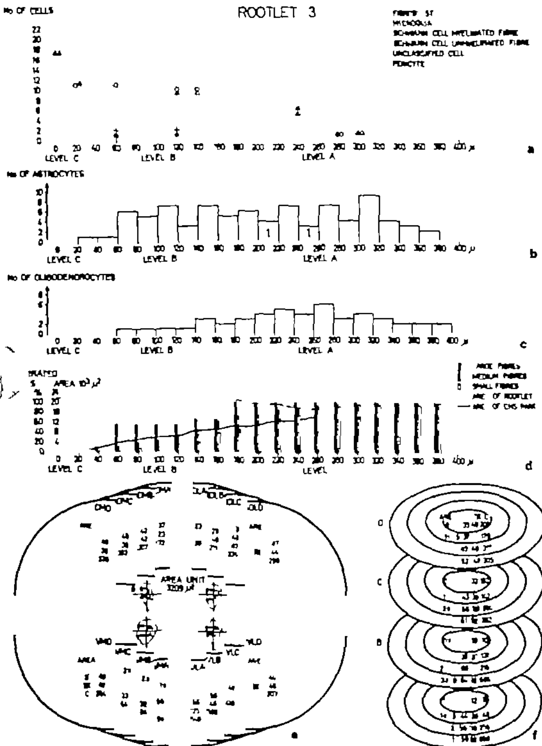
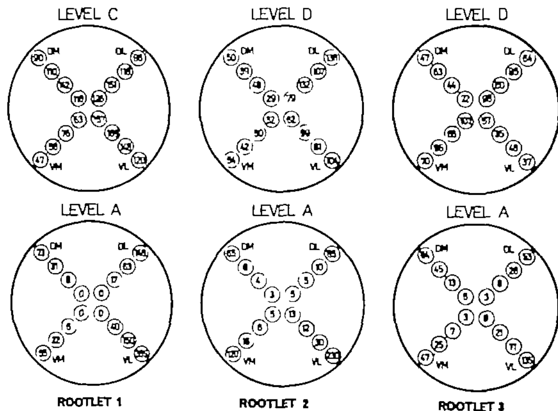

Fig. 23
ROOTLET 3
No OF CELLS
LEVEL C
LEVEL B
LEVEL A
No OF ASTROCYTES
No OF OLIGODENDROCYTES
AREA
AREA UNIT
a
b
c
d
e
f

Fig 24



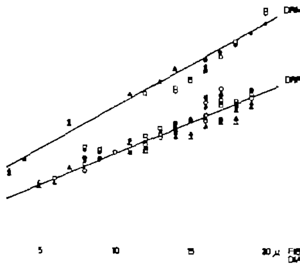
LEVEL C
LEVEL D
LEVEL D
LEVEL A
LEVEL A
LEVEL A
DM
DL
VM
VL
ROOTLET 1
ROOTLET 2
ROOTLET 3

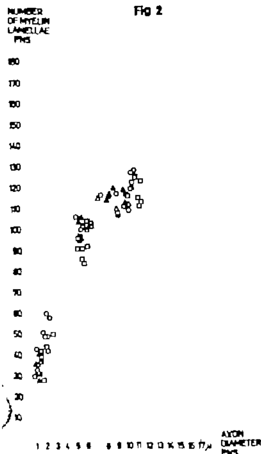
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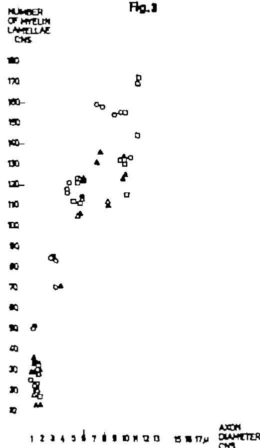
Observations on the morphology at the transition between the peripheral and the central nervous system in the cat. III Myelinated fibres in S_1 dorsal rootlets

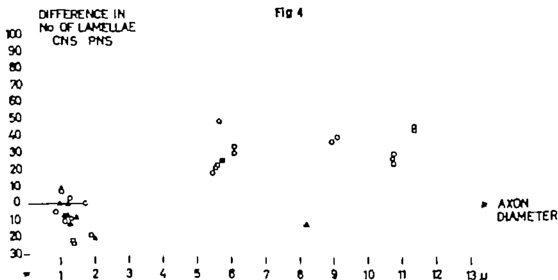
Running title Myelinated fibres in the transitional region

Myelinated fibres in the transitional region




Fig 2
NUMBER OF MYELIN LAMELLAE PNS
AXON DIAMETER PNS


Fig.3
NUMBER OF MYELIN LAMELLAE CNS
AXON DIAMETER CNS


Fig 4
DIFFERENCE IN No OF LAMELLAE CNS PNS
AXON DIAMETER

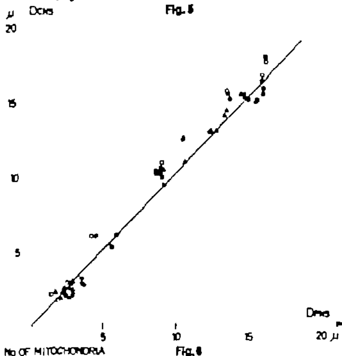


Fig. 5

No OF MITOCHONDRIA

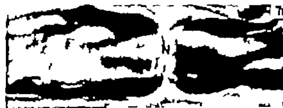
Fig. 6

400
300
200
100
10

100 90 80 70 60 50 40 30 20 10 0 10 20 30 40 50 60 70 μ

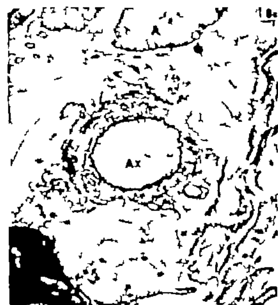
NoR


7
BN




GF
GF
Ax


Ax


A
Ax


Ax


BM


P
BN


P
BN
C

12

BN

12

P

BN

C

C


14a
PM
CM
Ax
14b
CM
PM
A
14c
CM
14d
CM
PM
CM
Ax
CM
CM

Figures to IV

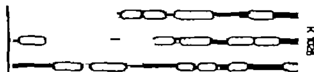
Observations on the morphology at the transition between the peripheral and the central nervous system in the cat. IV Unmyelinated fibres in S_1 dorsal rootlets

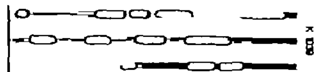
Running title. Unmyelinated fibres in the transitional region

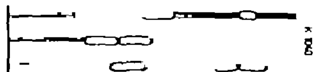
Unmyelinated fibres in the transitional region

Fig.2

PNS-CNS BORDER LINE 10 20 30 40 50 60 70 80 90 100 110 μ







PNS-CNS BORDER LINE 10 20 30 40 50 60 70 80 90 100 110 μ















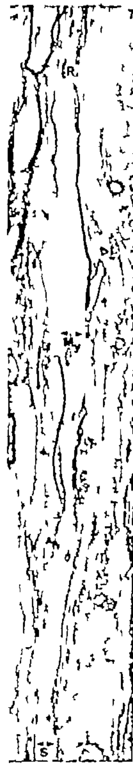







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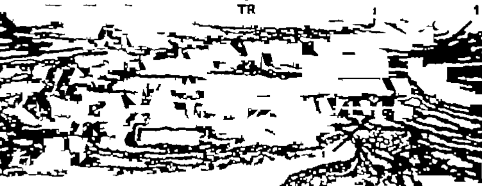




Figures to V

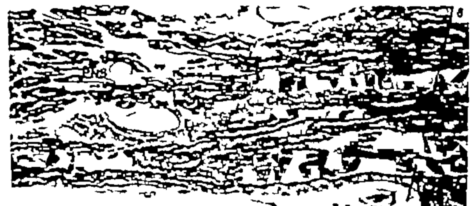
Observations on the morphology at the transition between the peripheral and the central nervous system in the cat. V A light microscopical and histochemical study of S_1 dorsal rootlets in developing kittens

Running title: Development of the transitional region


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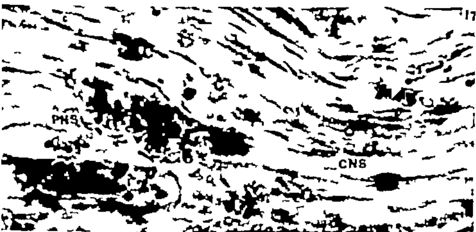

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ACTA PHYSIOLOGICA SCANDINAVICA
Supplementum 4[illegible]

Glomerulotubular balance in the rat kidney

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LÆGEFORENINGENS FORLAG

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ACTA PHYSIOLOGICA SCANDINAVICA
Supplement 447

Glomerulotubular balance in the rat kidney

Forsvaret finder sted torsdag den 26. maj 1977 kl. 14 præcis i Annexauditorium A, Studiestræde 6 o.g., København.

To Gunilla

SUMMARY OF INVESTIGATIONS

The present study was designed to increase our knowledge of certain aspects of glomerulotubular balance.

1 The relationship between serum protein concentrat ion and colloid osmotic pressure was measured in the rat and found to be in complete agreement with the Landis-Pappenheimer formula derived from measure ments made with human plasma. Establishment of the validity of this formula permitted further calculations to be made.

2 A mathematical analysis of the hydrodynamics of the renal corpuscle was used to quantify under steady stat conditions, the influence of changes in the following variables on glomerular filtration rate, nephron blood flow and on hydrostatic pressure in the glomerular capillaries

I arterial haematocrit and plasma protein concentrat ion,
II hydrodynamic resistance of both the afferent and efferent arterioles
III ultrafiltration coefficient of the glomerular membrane;
IV hydrostatic pressure in the proximal tubule
V hydrostatic pressure in the peritubular capillaries;
VI arterial blood pressure.

Nephron GFR was predicted to be very sensitive to the concentration of arterial plasma protein. The calculated change in glomerular capillary pressure accompanying a decrease in nephron GFR caused by either an increase in the afferent or decrease in the efferent arteriolar resistance to blood flow agreed with experimentally obtained values. The effect on nephron GFR of changing the hydrostatic pressure in the proximal tubule was calculated and found to agree with the experimental measurements described below

3. The influence of intratubular flow rate on rate of proximal fluid reabsorption (RPFR) was studied using free flow micropuncture techniques. By introducing a small decrease in proximal tubular hydrostatic pressure, nephron GFR was increased. The resulting increase in tubular flow rate was not accompanied by any change in RPFR.

4. The function of proximal convoluted tubules was studied after infusion of angiotensin II into adjacent peritubular capillaries. Infusion of angiotensin II resulted in a substantial reduction in RPFR which was associated with a significant increase in hydrostatic pressure in both the adjacent proximal tubules and the infused capillaries.

5. Similar peritubular infusion experiments were carried out using an antagonist to angiotensin II. The results of these experiments indicated that angiotensin II is a physiological regulator of hydrostatic pressure in the proximal tubule and the peritubular capillaries. No evidence was found of a tonic influence of angiotensin II on RPFR.

Full details of the techniques employed in the micropuncture studies are elaborated in papers 3, and 5

GLOMERULOTUBULAR BALANCE IN THE RAT KIDNEY

A nearly constant ratio has been measured between the glomerular filtration rate (GFR) and the rate of fluid reabsorption in the proximal tubule (RPFR), despite the occurence of large variations in GFR. This ratio, usually termed glomerulotubular balance, remains almost constant regardless of whether the changes in GFR and RPFR occur spontaneously in different nephrons of the same rat or are induced in the same nephron by aortic constriction, renal venous constriction or ureteral obstruction (1 2, 3, 4 5). The spontaneous tubule to tubule variations in GFR and RPFR do not, therefore, appear to be caused by differences in glomerular size and tubule length. Furthermore, the hydrostatic pressure in the proximal tubule generally varies very little regardless of large variations in GFR (6, 7 8, 9 10, 11).

Accordingly evaluation of the physiological mechanism of glomerulotubular balance requires an examination of the variables influencing GFR, RPFR and hydrostatic pressure in the proximal tubule. For this purpose, it is instructive to make a mathematical analysis of the hydrodynamics of nephron function, and to compare predicted values with experimental results.

Hydrodynamics of nephron function

The decreases in hydrostatic pressure along the proximal and distal tubules are very small (6, 12, 13, 14 15) leaving only three effective sources of resistance to flow in each nephron

glomerular resistance; loop of Henle resistance; and collecting duct resistance. For the total number of nephrons within the kidney each of these three resistances is connected in parallel. By analogy with the vascular system, the nephrons within the kidney may logically be regarded as comprising a branched tubular system with respect to their hydrodynamic characteristics. Mathematical analysis may then be simplified by

the concept of »the equivalent« nephron: a hypothetical single nephron which has the same hydrodynamic characteristics as the entire population of nephrons within the kidney

For the equivalent nephron, the pressure head which drives fluid against the nephron resistance, i.e. the glomerular propulsive pressure (16), is equal to the difference between the mean hydrostatic pressure in the glomerular capillaries (less the mean glomerular oncotic pressure) and the pelvic hydrostatic pressure. Equivalent glomerular resistance is found by dividing the glomerular resistance of the single nephron by the total number of nephrons within the kidney approximately 30,000 in the rat (17 18). Equivalent Henle loop resistance is obtained similarly Collecting duct resistance has been shown to be located mainly in the terminal collecting duct (19, 20). Assuming that the collecting duct resistance is exclusively terminal, an approximate value for the equivalent collecting duct resistance is given by dividing the resistance of the single collecting duct by the number of terminal collecting ducts in the kidney approximately 20 in the rat (18).

In sofar as all nephrons are assumed to function in an identical manner these calculations involve oversimplification. But they do provide an appropriate first approximation as indicated by the recent finding that the hydrostatic pressure in the loop of Henle of the juxtamedullary rat nephron is similar to that of the superficial nephron (19).

From experimental measurements in the hydropenic rat (6, 13, 19 21 22, 23, 24 25 26), the following values may be derived. equivalent glomerular resistance 5×10^{-4} mm Hg nl^{-1} min; loop of Henle resistance 15×10^{-4} mm Hg nl^{-1} min; collecting duct resistance 700×10^{-4} mm Hg nl^{-1} min. The hydrodynamic resistance of the glomerular membrane is thus very small compared with distal tubular resistance.

An increase in GFR is known to increase the urine flow rate, but the percentage of additional filtered fluid reabsorbed will be smaller than the percentage of fluid reabsorbed previously Taking this finding together with the calculated distributio of hydrodynamic resist

ance within the nephron gives the equivalent nephron definite characteristics (16, 27 28). Hydrostatic pressure in the proximal tubule will be relatively insensitive to changes in RPFR but will be markedly affected by the most modest pertubations in the glomerular propulsive pressure. Conversely GFR may be considerably chang ed by quite small alterations in RPFR while being very insensitive to changes in glomerular propulsive pressure. Any reduction in the tubular resistance will lead to a considerable increase in GFR and urine flow

An increase in RPFR will result in an almost identical increase in GFR, because the hydrostatic pressure required to drive an additional quantity of fluid across only the glomerular membrane is negligible in comparison with the pressure that would be required to drive a similar quantity of fluid across the resistance of the entire nephron. It follows that the flow of fluid which overcomes the total nephron resistance, i.e. the urine flow rate, and the hydrostatic pressure in the proximal tubule will be only slightly affected by changes in RPFR. Even if dramatic changes are induced in RPFR, the effect on the proximal intratubular pressure will be negligible.

Increasing the glomerular propulsive pressure will increase GFR and urine flow rate, but only a small proportion of the additional fluid filtered will be reabsorbed in the tubules. A large part of the additional fluid filtered must consequently overcome the entire nephron resistance, which is much greater than the average resistance to fluid being reabsorbed. Accor dingly the increment in glomerular propulsive pressure required to induce an increment of 1 ml min^{-1} in GFR will be greatly in excess of the unit pressure head previously required, i.e. a given percentage increase in glomerular propulsive pressure results in a much smaller percentage increase in GFR. The increase in hydrostatic pressure in the proximal tubule will be almost as much as the rise in glomerular propulsive pressure, since nephron resistance derives mainly from the distal part of the nephron.

One may conclude that glomerulotubular balance — the nearly constant ratio found to exist between GFR

and RPFR — merely expresses the fact that GFR cannot change significantly unless some alteration occurs in RPFR, assuming tubular resistance to be constant.

Comparison of predicted values with experimental results

Experiments have shown that decreasing the arterial blood pressure below the level of renal autoregulation in the hydropenic rat results in a reduction in the glomerular capillary pressure (9 11 29). This reduction was associated with decreased renal blood flow and hence a reduced glomerular propulsive pressure in the superficial nephron (9 11 29). The percentage decrease in glomerular capillary pressure was considerably smaller than the concurrent reduction in arterial blood pressure (9 11 29). Hydrostatic pressure in the proxi mal tubule remained almost constant (7 9 11 29). Accordingly a substantial reduction in nephron GFR was recorded (9 29), and RPFR decreased in proporti on to the reduction in nephron GFR (29, 30, 31). Hydrostatic pressure in the distal tubule decreased and there was a substantial increase in the collecting duct resistance (7).

EVALUATION OF INTRATUBULAR PRESSURE

Stabilization of the hydrostatic pressure in the proximal tubule must involve changes either in the glomerular propulsive pressure or in the tubular resistance, since the foregoing analysis of nephron hydrodynamics shows that changes in RPFR will only have a very small effect on intratubular pressure. Thus the near constant proximal intratubular pressure observed during aortic constriction, despite a substantial decrease in glomerular propulsive pressure, must have resulted from an increase in tubular resistance. Stabilization of the hydrostatic pressure in the proximal tubule by this mechanism results in a substantial decrease in the flow rate to the distal tubule and in the urine flow rate. These events lead to a reduced loss of body fluid and may serve to maintain the extracellular fluid volume during hypot nsion. Conversely the decrease in tubular

resistance observed during salt loading (20, 24) may increase sodium and water excretion and restore fluid homeostasis.

Stabilization of the proximal intratubular pressure results mainly from changes in collecting duct resistance, since the resistance of the loop of Henle constitutes only a small part of the tubular resistance. The mechanism proposed for stabilizing the intratubular pressure requires that the tubular resistance is very sensitive to the transmural hydrostatic pressure gradient. The loop of Henle and the collecting duct have a resistance to flow which has been shown to be very sensitive to the intratubular pressure within the physiological range of transmural pressures. These experiments have involved in vitro measurements of pressure, flow rate and tubular diameter relationships in thin descending limbs of Henle (32), in vivo recordings of pressure and flow in perfused superficial loops of Henle (25, 26), and pressure measurements in the distal tubule and the inner medulla under free flow conditions (20).

Hydrostatic pressure in the proximal tubule during aortic constriction is therefore stabilized mainly by an increase in the tubular resistance. Additionally the results of several studies provide evidence for the existence of a mechanism regulating glomerular propulsive pressure that involves an intrarenal feedback mechanism which stabilizes the supply rate of sodium chloride to the distal tubule. The juxtaglomerular apparatus, having both vascular and tubular constituents, is well disposed to play a role in such a feedback mechanism for compensatory adjustments between glomerular and tubular functions (33, 34, 35). Renin in the juxtaglomerular apparatus could serve to mediate these adjustments with renin activity determining the local formation rate of the potent vasoconstrictor angiotensin II (7, 36, 37, 38, 39, 40, 41). Angiotensin II might control the glomerular propulsive pressure (7, 36, 37, 38, 39, 40, 41, 42, 43) and so stabilize the flow rate to the distal tubule.

Evidence for the existence of this feedback mechanism derives from experiments in which the loop of Henle of the superficial nephron was perfused with a

capillaries (59) (paper V). An angiotensin II antagonist was infused into the peritubular capillaries and hydrostatic pressure was measured both in the infused capillaries and the adjacent proximal tubules. Infusion of this antagonist produced a decrease in hydrostatic pressure in both the capillaries and the proximal tubules; the most striking fall occured in rats with relatively high levels of plasma renin activity (PRA).

Summary

The increase in tubular resistance accompanying a reduction in arterial blood pressure below the level of renal autoregulation decreases renal fluid excretion and may serve to maintain the extracellular fluid volume. The decrease in tubular resistance associated with extracellular volume expansion may contribute to the maintenance of body fluid homeostasis. Glomerular propulsive pressure has been shown to be regulated by an intrarenal feedback mechanism. This mechanism is capable of depressing the glomerular propulsive pressure when the flow rate to Henle's loop is increased above that of normal but produces no change when the flow rate is reduced below normal. Feedback response is lowered during expansion of the extracellular fluid volume. The feedback mechanism appears to prevent overelevation of the flow rate in relation to the reabsorptive capacity of the distal tubule and collecting duct hence potentially dangerous losses of salt and water are prevented. For example, circulatory changes during feeding and muscular activity may be expected to change the glomerular propulsive pressure with the result that flow rate to the distal tubule increases. In addition, the intratubular pressure change accompanying increased glomerular propulsive pressure may possibly lead to a reduction in tubular resistance, producing a further increase in fluid excretion.

EVALUATION OF RPFR

The observed decrease in RPFR accompanying the reduced GFR during aortic constriction might be either a direct consequence of decreased GFR or related to changes in the peritubular concentration of some

humoral substance. A slight reduction in GFR might possibly induce a decrease in RPFR, which in turn would result in a further reduction in GFR, i.e. a positive feedback loop. Such an induced decrease in RPFR might be a consequence of an intrinsic or extrinsic tubular mechanism.

INFLUENCE OF TUBULAR FLUID FLOW RATE ON RPFR

The influence of flow rat on RPFR has been examined in several studies employing in vivo and in vitro pump perfusion of individual segments of proximal convolut ed tubules. Most of these studies have shown very little or no influence of perfusion rate on RPFR (60, 61 62, 63).

Nevertheless, a carefully executed study using plasma ultrafiltrate as perfusion fluid, showed that a 40 per cent decrease in perfusion rate produced a 17 per cent decrease in RPFR (64). In another study aspirating fluid at an early proximal site reduced the flow rate without influencing nephron GFR (65). A 45 per cent reduction in flow rate of tubular fluid was associated with an average reduction of 29 per cent in RPFR. On the other hand, aortic constriction sufficient to decrease the filtration rate by 50 per cent has been shown to reduce RPFR by 50 per cent in pump perfused segments of proximal tubules perfused at a constant flow rate throughout (66). Furthermore, another study (10) (Paper III) indicates that an increase in nephron GFR induced by a decrease in hydrostatic pressure in the proximal tubule does not change RPFR.

Although these results are inconsistent with respect to the magnitude of the change in RPFR induced, they do indicate that a change in the flow rate of tubular fluid is associated with a significant change in the fractional reabsorption of tubular fluid in the proximal tubule. Accordingly changes in the intratubular flow rate alone appear unlikely to account for th balance between filtration rate and proximal reabsorption observed during aortic constriction.

An increase in GFR could conceivably alter RPFR by changing the filtered amount of glucose, amino acids

and bicarbonate, since a proportion of the tubular sodium reabsorption is known to be coupled to the transport of these solutes (67 68, 69, 70, 71 72, 73, 74 75, 76). The concentrations of organic solutes in the ultrafiltrate are low so that only bicarbonate may significantly affect the reabsorption of fluid under physiological conditions.

A mathematical analysis of flow through folded membrane structures has shown that the degree of stirring within the folds may affect the solute concentration profiles within the folds, resulting in altered solute flow through the membrane (77). An increase in the flow rate of tubular fluid has been proposed to increase mixing of the unstirred diffusion barrier within the crypts between the microvilli (65). This mixing might in turn augment the sodium concentration in the diffusion barrier and enhance sodium diffusion into the cells, with the result that sodium reabsorption increases. This theory is fundamentally similar to that of Kelman (78) who suggested that RPFR is proportional to the flow rate of tubular fluid.

EFFECT OF PROTEIN COLLOID OSMOTIC PRESSURE ON RPFR

A decrease in GFR might cause RPFR to decrease under the influence of a mechanism extrinsic to the tubule, for instance, by reducing the peritubular protein concentration. Experiments indicate that aortic constriction reduces the filtration fraction in the superficial rat nephron (9 29 79)· this will reduce the protein concentration and hence the plasma colloid osmotic pressure in the efferent arteriole.

The influence of colloid osmotic pressure on RPFR has been studied extensively employing both in vitro and in vivo micropuncture techniques. In vitro studies using microperfused fragments of rabbit proximal convoluted tubules have shown that changes in the ambient protein colloid osmotic pressure exert a marked effect on RPFR even when the capillary network is absent (80, 81 82). This interpretation has been challenged by the results of a similar in vitro microperfusion study which suggested that the effect

on RPFR ascribed to the protein colloid osmotic effect per se was actually caused by unidentified protein accelerators and inhibitors of tubular fluid transport (83).

In vivo studies have been carried out employing either intraluminal application or perfusion of the peritubular capillaries with solutions of differing colloid osmotic pressure. In the first type of experiments RPFR was derived from pump perfusion of tubular segments (84). An effective hydraulic conductivity L_e of 0.05 nl min^{-1} mm tubule length^{-1} mm Hg^{-1} was recorded in these experiments.

In the experiments with peritubular perfusion, RPFR in the adjacent proximal convoluted tubules was derived from simultaneous pump perfusion of individual tubular segments (85), from recordings of the t value for the disappearance of a droplet of saline splitting an oil column within the tubule (shrinking droplet technique) (86, 87), or from timed quantitative collections of fluid from the end of the proximal convolution (88, 89, 90, 91).

A change in RPFR of 0.03 nl min^{-1} tubule length^{-1} mm Hg^{-1} was found from experiments in which RPFR was estimated by simultaneous tubular microperfusion or by the shrinking droplet technique (85, 86, 87).

The experiments involving peritubular capillary microperfusion and simultaneous fluid collection from the end of the proximal convolution have given inconsistent results (88, 89, 90, 91). Brenner et al found an increase in RPFR of about 0.05 nl min^{-1} mm tubule length^{-1} mm Hg^{-1} when th peritubular capillary protein concentration was increased above the control level by vascular microperfusion during aortic constriction (89). According to this study approximately 40 per cent of the decrease in RPFR observed during aortic constriction could be attributed to the associated reduction in peritubular protein concentration. In contrast, Conger et al (88) found RPFR unaffected by varying the peritubular protein concentration between zero and 13 g %.

Similar absence of response was obtained in another carefully executed study (90, 91), nephron GFR

and RPFR were recorded in the same tubules, before and during aortic constriction, employing the re collection micropuncture technique. In one group of nephrons, the peritubular capillary flow rate and protein concentration was kept constant by vascular microperfusion prior to and during aortic constriction. Recordings from these tubules were compared with those obtained from control tubules without vascular microperfusion in which peritubular flow rate and protein concentration were allowed to change. Similar changes in nephron GFR and RPFR were induced in both groups of tubules, suggesting that intraluminal factors mediate the decrease in RPFR during aortic constriction. As mentioned above (page 17) contrasting results have been obtained (66).

Colloid osmotic pressure of efferent arteriolar blood in the superficial rat nephron has been found to decrease by 6 mm Hg associated with a reduction in nephron GFR of 8 nl min^{-1} during aortic constriction, with a decrease in the femoral artery pressure from 120 to 80 mm Hg (9). Given the assumption that the TF/P inulin value of 2.5 was unchanged, RPFR would have decreased by 4.8 nl min^{-1}. With a tubular length of 5 mm and a change in RPFR of 0.05 nl min^{-1} mm tubule $length^{-1}$ mm Hg colloid osmotic $pressure^{-1}$ the decrease in efferent arteriolar protein concentration would have resulted in a reduction in RPFR of 1.5 nl min^{-1} which comprises only 31 per cent of the expected decrease in RPFR. Reduction in the peritubular protein concentration therefore appears unlikely to account entirely for the decrease in RPFR during aortic constriction. Nevertheless, the influence of protein colloid osmotic pressure on glomerulotubular balance remains a controversial subject.

EFFECT OF HUMORAL SUBSTANCES ON RPFR

The further possibility exists that the decrease in RPFR associated with aortic constriction is controlled humorally. The diminution in flow rate of tubular fluid to the distal tubule during aortic constriction has been proposed to activate a feedback mechanism resulting in an altered peritubular concentration of a humoral substance

which influences RPFR (3). Angiotensin II has been proposed as a messenger in this feedback mechanism (37). The effect of angiotensin II on RPFR has been the subject of much controversy.

In vivo microperfusion studies have produced evidence against an individual nephron feedback mechanism. Proximal tubules were perfused from an early proximal site and the flow rate to the distal tubule was altered, either by making partial collections of fluid at the end of the proximal convolution (60) or by changing the pump perfusion rate (61). These experiments showed that RPFR in the perfused segments of the proximal tubules did not change when the flow rate to the loop of Henle was altered.

No effect of angiotensin II on RPFR was found during intravenous infusion of subpressor doses of angiotensin II in experiments where the RPFR was determined from timed collections of tubular fluid (92, 93) or by using the shrinking droplet technique (94). Similarly experiments using solutions with very high angiotensin II concentrations administered by intratubular application (94, 95) or peritubular perfusion (95) were without effect. Finally RPFR was not affected by angiotensin II in the in vitro perfused proximal tubule (63).

Contrasting results were obtained from experiments in which the occlusion time (the time for complete collapse of the proximal tubular lumen after clamping the renal artery) was used as a measure of RPFR. In these experiments a decrease in RPFR was found after intravenous injections of angiotensin II (96). A marked inhibition of RPFR was also found in a later study employing peritubular infusion with relatively high physiological concentrations of angiotensin II (97) (paper IV). RPFR was derived from timed collections of tubular fluid from the end of the proximal convoluted tubule.

In an attempt to determine whether angiotensin II exerts a tonic influence on RPFR, similar peritubular infusion experiments were carried out with an analogue of angiotensin II 1-sarcosine, 8-alanine angiotensin II (59) (paper V). This analogue was found to block the

effect of exogeneous angiotensin II on RPFR, but peritubular infusion with the angiotensin II analogue did not increase RPFR. These results fail to provide evidence for a tonic influence of angiotensin II on RPFR.

Summary

The decrease in RPFR during aortic constriction could occur either as a direct consequence of the decrease in GFR or be induced by a humoral substance. A decrease in GFR might influence RPFR by an extrinsic or an intrinsic tubular mechanism. An intrinsic mechanism has been excluded by experiments which demonstrate that altering the flow rate of tubular fluid induces a change in RPFR which is too small to account entirely for the glomerulotubular balance observed during aortic constriction. A mechanism extrinsic to the tubule also appears unlikely to be responsible for glomerulotubular balance experimental results indicate that the decrease in RPFR caused by a measured reduction in peritubular protein oncotic pressure during aortic con striction is inadequate for this purpose. Angiotensin II has been proposed to mediate changes in RPFR during aortic constriction. Experimental results have not provided evidence of a tonic influence of angiotensin II on RPFR. An as yet unidentified humoral substance may be shown to be responsible for glomerulotubular balance. Alternately glomerulotubular balance may be the result of contributions from all of the above mentioned variables.

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Copenhagen, May 1976.

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ACTA PHYSIOLOGICA SCANDINAVICA
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Aspects of Cardiovascular Reflex Control in Man

An experimental study

BY

GUNNAR TYDÉN

STOCKHOLM 1977

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Faculty of Medicine, Karolinska Institutet,
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IV Bjurstedt H G Rosenhamer and G Tydén Lower body negative pressure and effects of autonomic heart blockade on cardiovascular responses Acta physiol scand 1977 99 353-360

In the text these papers will be referred to by their Roman numerals I-IV

PREFACE

The present investigation was performed at the Department of Environmental Physiology Karolinska Institutet Stockholm The experimental part of the investigation was carried out during the years 1972-1975

I wish to express my profound thanks to Professor Hilding Bjurstedt Head of the Department of Environmental Physiology who introduced me into the field of cardiovascular research He has generously shared with me his experience in this field and has given me continual support throughout this study

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INTRODUCTION

Since the demonstration by Hering (1923) of the baroreceptor reflex function of the carotid sinuses knowledge about the physiology of the reflex control of circulation has accumulated at an ever increasing rate The methods of study of baroreceptors and baroreceptor reflexes have been described and summarized in numerous publications Notable is the monograph of Heymans and Neil (1958) which provided a much needed full discussion of the role of baroreceptor reflexogenic zones in the control of circulation Integrated aspects of cardiovascular reflex control have been presented in more recent reviews and monographs e g those of Folkow Heymans and Neil (1965) Henry and Meehan (1971) Korner (1971) Kirchheim (1976) and Öberg (1976)

The information pre ently available on cardiovascular reflex control has predominantly been derived from experiments performed on anesthetized animals where it has been possible to study separate reflex mechanisms while inputs from other afferent zones have been maintained constant or eliminated (for review see Heymans and Neil 1958) Even though such experiments are basic to any thorough analysis of circulatory control they must be followed by studies on intact conscious man It is well known that anesthesia as well as surgical trauma can alter the responses of the cardiovascular system to a variety of physiologic stimuli (for recent re-

views see Vatner and Braunwald 1975 Kirchheim 1976) A number of direct and indirect methods have therefore been developed to make possible the study of baroreceptor reflex function in unanesthetized man A physiological and simple way to challenge circulatory homeostasis familiar to all physiologists is to tilt a subject from the supine to the head-up position Indeed many reflex mechanisms involved in postural changes in the circulation have been studied extensively and have been aptly reviewed by Gauer and Thron (1965) To achieve more clearcut but still reversible circulatory responses the gravitational pull in the head-foot direction can be augmented by exposure to increased gravitational force on the human centrifuge The baroreflex involvement in cardiovascular adaptation to accelerative stress has been reviewed by Gauer and Zuidema (1961) and Howard (1965)

Circulatory reflexes evoked by a shift from the recumbent to the head-up position are due mainly to arterial hypotension in the upper portion of the body and to a downward displacement of blood volume from the intrathoracic space Such reflexes can be studied separately The reflex effects on the heart of a drug-induced decrease in arterial pressure have been studied in the supine as well as the erect posture (Robinson et al 1966 Pickering et al 1972a b) However the vasoconstrictor component of the baroreceptor reflex cannot easily be evaluated by this method of drug-induced arterial hypotension since it is masked by the direct effect of depressor drugs on vascular smooth muscle A more physiological method of changing the stimulus to the carotid sinus barore

ceptors in man was first devised by Ernsting and Parry (1957) who increased the carotid sinus transmural pressure by decreasing the pressure in an airtight box enclosing the neck This method having subsequently been employed in several investigations (Bevegård and Shepherd 1966 Beiser et al 1970 Eckberg et al 1975 1976 Eckberg 1976) was further developed by Thron et al (1967) to permit also the application of positive pressure on the neck thus simulating carotid sinus hypotension (Wagner et al 1968 Stegemann Busert and Brock 1974) Finally the effects of direct carotid sinus nerve stimulation have been studied in anesthetized (Carlsten et al 1958) and conscious man (Epstein et al 1969 Eckberg Fletcher and Braunwald 1972) It must be borne in mind however that the effects of electrical stimulation of the sinus nerve result from both chemoreceptor and baroreceptor nerves which complicates their interpretation

The circulatory responses to a decrease in intrathoracic blood volume can be studied with the body in the supine position by exposing its lower portion to subatmospheric pressure This method of applying lower body negative pressure (LBNP) to simulate certain circulatory effects of gravity was first devised by Greenfield et al (1963) and may produce considerable pooling in dependent veins with little if any effects on the arterial blood pressure at head level (for review see Wolthuis Bergman and Nicogossian 1974) Negative pressures of 40-50 mm Hg have been used to produce physiological changes comparable to those observed during orthostasis and head-up tilt (Gilbert and Stevens 1966

Samueloff Browse and Shepherd 1966 Murray et al 1968 Wolthuis Hoffler and Johnsson 1970 Musgrave Zechman and Mains 1971 Rowell et al 1972) Indeed pressures of this magnitude have been extensively used in many space missions to simulate the effects of normal gravity on the body in the weightless condition (for review see Pestov and Gerathewohl 1975) Few investigations have involved the application of negative pressures of greater magnitude (Brown et al 1966 Stevens and Lamb 1965)

Since few of the above-mentioned investigations have involved the simultaneous measurement of cardiac output and arterial pressure little is known about the relative contributions of cardiac output and total peripheral resistance in the cardiovascular reflex control in man Thus the effects of increased gravitational force acting in the head--seat direction on cardiac output and total peripheral resistance have previously only been studied by Lindberg et al (1961) who found that exposure to increased gravitational force in the sitting position elevated mean arterial pressure at heart level due to a marked increase in total peripheral resistance while cardiac output was only moderately decreased

Ernsting and Parry (1957) using the direct Fick method and Bevegård and Shepherd (1966) employing the dye dilution technique examined the effects of increased carotid sinus transmural pressure on cardiac output and arterial pressure Ernsting and Parry (1957) found no significant effect on cardiac output; the reduction in arterial pressure evoked by

increased carotid sinus transmural pressure was therefore attributed to vasodilatation probably in the splanchnic area Bevegård and Shepherd (1966) on the other hand who noted a significant decrease in cardiac output when negative pressure was applied to the neck suggested that the decrease in systemic arterial pressure with carotid sinus stimulation results from a slight decrease in cardiac output and some dilatation of resistance vessels Electrical stimulation of the carotid sinus nerves by means of an implanted stimulator was found to reduce mean arterial pressure by reflexly decreasing both vascular resistance and cardiac output in the work of Epstein et al (1969) whereas only cardiac output was reduced in the study of Farrehi (1972)

So far no information is available as to the effect of decreased carotid sinus transmural pressure on the cardiac output in man However Roddie and Shepherd (1957) examined the effects of compression of the carotid arteries below the level of the carotid sinuses on the vascular resistance in the limbs Since vascular resistance in the forearm calf and hand were unchanged or slightly decreased during the increase in arterial pressure elicited by carotid artery compression these authors concluded that the pressor response was due to increased cardiac output or increased peripheral resistance in other vascular beds or both

Measurements of cardiac output and arterial pres ure during lower body negative pressure have previously been carried out by Steven and Lamb (1965) Murray et al (1968) and Ro-well et al (1972) It was observed that LBNP may produce a

marked decrease in cardiac output with mean arterial pressure essentially unchanged due to a corresponding increase in total peripheral resistance In the study of Rowell et al (1972) it was concluded that approximately a third of the increase in total vascular resistance was due to splanchnic vasoconstriction Stevens and Lamb (1965) and Murray et al (1968) extended the exposures to LBNP until the occurrence of presyncopal signs or vasodepressor syncope respectively and found that the fall in mean arterial pressure associated with impending syncope is primarily due to a fall in systemic vascular resistance It was concluded by Stevens and Lamb (1965) that the individual s ability to maintain his blood pressure through peripheral vascular mechanisms determines the course of events during exposure to LBNP

Following the introduction of selective beta-adrenergic blocking agents the use of such agents and of atropine has made it possible to pharmacologically isolate the heart from sympathetic or parasympathetic influence or from both This opportunity has been utilized to study in conscious man the relative roles of the two segments of the autonomic nervous system in the baroreflex control of the heart rate in various conditions In such studies the tonic restraint exercised from the carotid sinus reflexogenic areas has been deliberately altered by applying some of the methods described above Likewise autonomic cardiac blockade has been utilized to study the relative roles of cardiac and peripheral effector mechanisms in certain conditions such as hypertension (Julius et al 1971 Ellis and Julius 1973 Korner et al 1973) exercise (Cummings and

Carr 1966 Nordenfelt 1971) and the Valsalva maneuver (Korner Tonkin and Uther 1976) However it seems that beta-adrenergic or combined autonomic cardiac blockade has so far not been utilized to study the relative importance of cardiac effector mechanisms in the circulatory adaptation to such conditions as increased gravitational force bidirectional changes in the carotid sinus transmural pressure or lower body negative pressure

The objectives of the present investigation were to study in conscious man the function of certain cardiovascular reflex mechanisms in experimentally induced disturbances of circulatory homeostasis Such disturbances were brought about by exposing subjects to different experimental conditions viz 70^{o} head-up tilt (II) a threefold increase of the force of gravity (I III) bidirectional changes in the carotid sinus transmural pressure (II) and lower body negative pressure (IV) In this way it has been possible to study different reflex effects on the circulation primarily induced by changes in the transmural pressure at the level of the carotid sinuses and by reductions in central blood volume separately or in combination In order to elucidate the contributions of cardiac effector mechanisms to these effects the cardiovascular adjustments to the above-mentioned conditions were observed before and after beta-adrenergic and/or combined beta-adrenergic and parasympathetic blockade

METHODS AND PROCEDURES

Descriptions of the methods and procedures used in this investigation to challenge circulatory homeostasis and to suppress the participation of cardiac effector mechanisms in certain reflex responses to such challenge have been given in detail in papers I-IV and are therefore only briefly summarized below The experimental conditions to which the subjects were exposed in the separate studies are presented in Table I

Subjects In all 20 healthy physically well trained male volunteers were studied Several of them took part in more than one of the series of experiments Dimensional and functional data for the subjects have been reported in the individual papers The subjects were thoroughly acquainted with the experimental procedure before their informed consent was obtained The design of the investigation has been approved by the Ethical Committee of the Karolinska Institutet

Normal and increased gravitational force For studies on circulatory responses to the force of normal gravity a tilt table with a foot plate was used were subjects could be passively tilted from the supine to the 70^{o} head-up position To produce increased gravitational force the human centrifuge (radius = 7 4 m) at the Karolinska Institutet was used Fig 1 shows the position of the subject in the centrifuge

TABLE I Experimental conditions to which the subjects were exposed in the separate studies

Study	n	Type of tress applied	Blockade
II	6	70 head-up tilt	Beta adrenergi and parasympathetic
I	6	Increased gravitational force 3 G resting	Beta-adrenergic
I	6	Increased gravitational force 3 G exercise	Beta-adrenergic
III	8	Increased gravitational f rce 3 G resting	Beta-adrenergic and parasympathetic
II	8	Changes in carotid inus trans mural pressure $\pm$40 mmHg supine	Beta- drenergi and parasympatheti
II	8	Changes in carotid inus trans mural pressure $\pm$40 mmHg erect	Beta-adrenergic and parasympath ti
IV	8	Lower body negative pressure 80 mmHg	Beta-adrenergic
IV	8	Lower body neg tive pressur 80 mmHg	Beta adrenergic and parasympathetic

gondola The gondola was allowed to swing out freely during the centrifuge runs so that the resultant force was always acting in the head-to-seat direction The speed of rotation was chosen so as to produce a threefold increase in the gravitational force (3 G) this force being well tolerable for subjects which have been made accustomed to the subjective sensations of sustained accelerative stress The gondola was equipped with bidirectional communication facilities as well as with a television camera for continuous supervision of the subject The actual G-force as well as the circulatory variables studied were continuously monitored and recorded outside the centrifuge via a slip-ring arrangement To make leg exercise possible during the centrifuge runs the gondola accommodated an electrically braked cycle ergometer with the crank axis at the level of the gondola seat

Changes in carotid sinus transmural pressure The carotid arterial stretch receptors were exposed to different transmural pressures according to the method of Thron et al (1967) using a plexiglass helmet enclosing the neck and the head The subject breathed air at normal atmospheric pressure through a mouthpiece attached to a hole in the helmet by means of a short rubber tube (II: fig 1) The pressure within the helmet could be changed in either direction and was monitored continuously by a strain gage manometer The time to reach the desired pressure was kept at about 4 sec

Lower body negative pressure (LBNP) The device to produce LBNP consisted of a wooden box with an elliptical opening at one end in the main similar to that described by

Stevens and Lamb (1965) A rubber seal was provided at the opening which fitted hermetically around the waist at the level of the iliac crest The subject rested supine straddling a narrow padded saddle which provided support during the suction exposures The box was connected via a pressure regulator to a remotely located preevacuated low pressure chamber which made possible the application of graded negative pressure The time to reach the desired negative pressure was kept at about 30 sec A strain-gage manometer was used for continuous monitoring of the pressure in the box Temperature inside the box was also monitored continuously and was kept at ambient room temperature within ±0 5°C

Catheterization procedures For recording of arterial pressure one radial artery was entered percutaneously at the wrist by a small Teflon catheter (length = 7 cm; O D = = 1 15 mm; I D = 0 75 mm) For withdrawal of arterial blood during cardiac output determinations by cuvette densitometry (see below) the contralateral radial artery was catheterized in a similar way using a catheter of somewhat larger caliber (O D = 1 45 mm; I D = 1 05 mm) A venous catheter (length = = 30 cm; O D = 1 7 mm; I D = 1 2 mm) was introduced percutaneously into an antecubital vein and advanced so that its tip was located in the axillary vein

Cardiac output was determined by the indicator dilution technique using indocyanine green (Cardio-Green®) and a Waters dichromatic densitometer (Model X-300 in study I D-401 in studies II-IV) Dye injections as well as the subsequent withdrawal and reinfusion of arterial blood through

the densitometer cuvette were made by a remote-controlled injection system described elsewhere (Rosenhamer 1967 1968) The amount of dye injected for each determination was 1 ml of a 0 5% aqueous solution of indocyanine dye and the rate of withdrawal of blood from the radial artery through the cuvette was 20 ml/min In study IV dichromatic earpiece densitometry was used (Reed and Wood 1967) this method having shown satisfactory agreement with results obtained with arterial cuvette densitometry (Reed and Wood 1967 Schneider Cheikhzadeh and Müller 1967 Wallgren 1975) A dichromatic earpiece (Waters XE-302) was then attached to the ear after rubbing the pinna for one minute with rubrifacient ointment (Transvasin®) and was secured in position with adhesive tape The method for dye injection and the amount of dye injected was the same as described above

Arterial pressure at heart level was obtained by means of a differential strain-gage manometer (Statham P 23 B) one of the inputs being connected to a radial artery catheter and the other to a plastic tube taped onto the skin along the mid axillary line and filled with saline up to the level of the insertion of the fourth rib This technique afforded automatic compensation for the hydrostatic pressure difference between the heart and the fixed strain gage and thus permitted accurate recordings of the mean arterial pressure and its changes at heart level irrespective of changes in the G level or body position Mean arterial pressure was derived electronically from the arterial pressure recordings

Heart rate was obtained from chest electrodes and recorded

by means of a linear beat-to-beat cardiotachometer (Lindborg Wigertz and Ödman 1969)

Recordings All signals were displayed on a multichannel strip-chart recorder (Brush Mk 200) and recorded on magnetic tape (Ampex FR-100 C) for storage and subsequent detailed analysis of the variables under study In addition the dye--dilution curves were inscribed on an X-Y recorder (Hewlett-Packard 700 AM) at a paper speed of 4 mm/s

Pharmacological blockade Beta-adrenergic blockade was produced by the i v administration of 0 25 mg/kg of propranolol (Inderal®) To achieve parasympathetic blockade atropine sulphate was given i v in doses of 0 03 mg/kg (II--III) and 0 04 mg/kg (IV) respectively

General procedures The experimental conditions to which the subjects were exposed in the separate studies may evoke a considerable emotional impact which may influence the reflex circulatory responses Care was therefore taken to accustom the subjects to the subjective sensations of the circulatory stress to be applied During the course of 1-5 weeks preceding the day of the experiment several preliminary sessions were therefore conducted on separate days with each subject No experiment was performed before the subject reported that he was completely familiarized with the subjective sensations involved in the procedure Furthermore no appreciable apprehension was present during the actual experiment as judged from the subject s resting heart rate

After the catheteri ation procedures the subject was allowed at least 30 min of rest before any circulatory stress

was applied Following the administration of propranolol and/or atropine no measurements were made until 10 min had elapsed

The time of exposure to acceleration stress was 5 min (I) and 4 min (III) respectively to changes in carotid sinus transmural pressure 2 min and to LBNP 4 min

Calculations Cardiac output was determined from the dye dilution curves according to the method originally deviced by Kinsman Moore and Hamilton (1929) Heart rate and mean arterial pressure were averaged during the inscription of each indicator dilution curve Total peripheral resistance was calculated as mean arterial pressure (mm Hg) divided by cardiac output (ml/sec) The statistical significance of differences between mean values were evaluated by applying the t-test to the intraindividual differences (cf Fisher 1948)

RESULTS

The results are presented in four main sections dealing with circulatory responses to head-up tilt increased gravitational force changes in carotid sinus transmural pressure and lower body negative pressure respectively In each section the effects of beta-adrenergic and/or combined beta-adrenergic and parasympathetic blockade on the circulatory responses are presented Absolute and percentage changes refer to group mean values For detailed additional information the reader should consult the individual papers (I-IV)

Head up tilt (II)

Effects of a shift from the supine position to 70° head-up tilt (6 min) were studied in six subjects before and after combined cardiac effector blockade

Mean arterial pressure at heart level was slightly increased (7%) following change to the head up position this increase not being affected by autonomic blockade The increase in heart rate normally following head up tilt was almost completely abolished by the blockade Cardiac output was decreased by 51% when assuming the upright position after blockade as compared to 26% in the unblocked condition However due to a potent increase in total peripheral resistance (113%) (fig 2) the exaggerated curtailment of cardiac output after blockade was fully compensated

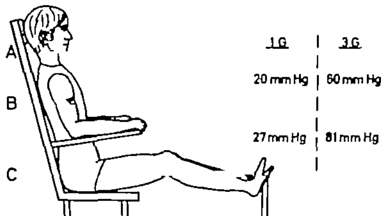


Fig 1 Position of subject in the centrifuge gondola with approximate hydrostatic pressure differences between the hydrostatic indifference point (B) the carotid sinuses (A) and the legs (C) respectively at normal gravity (1 G) and during a threefold increase in the gravitational force (3 G)

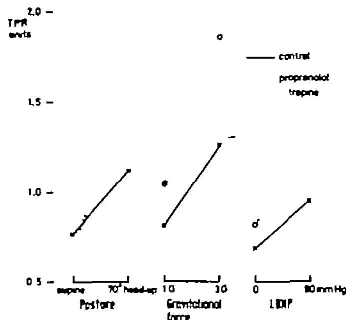


Fig 2 Changes in total peripheral resistance (TPR) induced by 70° head-up tilt (n=6) exposure to increased gravitational force (3 G n=8) and lower body negative pressure (LBNP n=8) before and after cardiac effector blockade

Increased gravitational force (I III)

Two separate experimental series were performed In the first six subjects were studied in the seated position before and during a 5 min exposure to a threefold increase in the gravitational force (3 G) Cardiovascular responses were investigated before and during leg exercise and before and after beta-adrenergic blockade The work load was preset individually to correspond to approximately 50% of the subjects aerobic working capacity The second series comprised eight subjects exposed to 4 min of 3 G before and after combined cardiac effector blockade

Exposure to 3 G in the sitting position produced an increase in mean arterial pressure at heart level from 99 to 126 mm Hg (I) and from 102 to 127 mm Hg (III) respectively Beta adrenergic blockade did not affect the blood pressure response to 3 G (95 to 125 mm Hg) which was however significantly decreased after combined cardiac effector blockade (104 to 118 mm Hg) During exercise exposure to 3 G caused an increase in blood pressure from 115 to 139 mm Hg before and from 105 to 130 mm Hg after propranolol (fig 3) G--induced increase in <u>heart rate</u> was 52 (I) and 45 (III) beats/min respectively Propranolol reduced this response by 62% whereas propranolol and atropine reduced it by 78% During exercise the G-induced heart-rate response was reduced by 92% by propranolol (fig 4) G-induced reductions in cardiac output were augmented by 25% after propranolol and by 30% after propranolol and atropine The absolute v lues during exposure to 3 G were 6 3 and 6 1 l/min (tudies

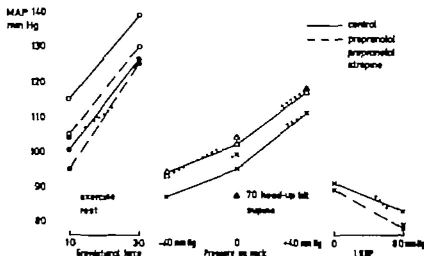


Fig 3 Changes in mean arterial pressure (MAP) at heart level induced by increased gravitational force (3 G) at rest (n=14 before blockade n=6 during propranolol n=8 during propranolol + atropine) and during exercise (n=6) by positive and negative pressures on the neck (n=8) and by lower body negative pressure (LBNP n=8) before and after beta-adrenergic and combined beta-adrenergic and parasympathetic blockade

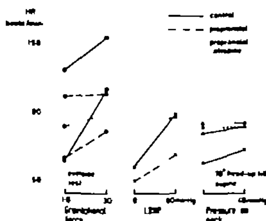


Fig 4 Changes in heart rate (HR) induced by increased gravitational force (3 G) at rest (n=14 *before blockade n=6* during propranolol n=8 during propranolol + atropine) and during exercise (n=6) by lower body negative pressure (LBNP n=8) and by pressure on the neck (n=8) before and after beta-adrenergic and combined beta-adrenergic and parasympathetic blockade

I and III) in the unblocked situation 4 2 l/min after beta-adrenergic and 3 9 l/min after combined beta-adrenergic and parasympathetic blockade Total peripheral resistance increased markedly in all conditions following exposure to 3 G (fig 2) Although the percentage increase was greater after combined blockade than after propranolol only the absolute values were almost identical 1 85 and 1 86 units respectively

Changes in carotid sinus transmural pressure (II)

Eight subjects were studied during 2 min periods of exposure to a 40 mm Hg increase or decrease respectively of the external pressure over the neck both in the supine and 70° head up position Six of the subjects participated in a second experimental session where the above-mentioned exposures were run after combined cardiac effector blockade

Positive pressure of 40 mm Hg on the neck produced an increase in mean arterial pressure of 16 mm Hg whereas negative pressure of the same magnitude caused a reduction in arterial pressure of only 8 mm Hg (fig 3) The arterial pressure responses obtained by changing the transmural pressure were essentially the same in the supine as in the 70° head-up position Furthermore combined autonomic blockade did not affect the reflex changes in mean arterial pressure in either position A 16% increase in heart rate accompanied the increase in mean arterial pressure during positive pressure on the neck in the supine position During head-up tilt the corresponding increase wa only 6% (fig 4) Heart-rate

response to negative pressure on the neck was -6% in the supine position but negligible in the head-up position Autonomic blockade drastically reduced changes in heart rate accompanying changes in carotid sinus transmural pressure although they were not completely extinguished Cardiac output increased significantly during positive pressure on the neck (10% in the supine as well as the head-up position) Autonomic blockade completely abolished this increase During negative pressure there were no changes in cardiac output neither before nor after blockade Whereas the arterial pressure response to increased carotid sinus transmural pressure was due solely to a reduction in total peripheral resistance a significant increase in cardiac output thus contributed to the more potent pressor response to decreased transmural pressure When the increase in cardiac output was abolished by autonomic blockade a compensatory increase in total peripheral resistance maintained the pressor response at the pre-blockade magnitude

Lower body negative pressure (IV)

Eight subjects were studied before and during 4 min periods of exposure to 80 mm Hg of LBNP before and after beta-adrenergic and combined beta-adrenergic and parasympathetic blockade

LBNP produced a slight decrease in mean arterial pressure towards the end of the exposure (9%) (fig 3) Propranolol augmented this decrease the absolute value at the end of the suction period being significantly lower than before blockade (78 mm Hg and 83 mm Hg respectively) After combined cardiac

effector blockade the pre-LBNP value of mean arterial pressure was significantly increased as compared to the unblocked condition and that with propranolol alone During LBNP however there was a clearcut reduction (21%) so that the value at the end of the period was the same as with propranolol alone Heart rate increased gradually during LBNP (+24 beats//min after 30 sec +39 beats/min at the end of the period) Propranolol reduced this response by 51% whereas combined blockade reduced it by 87% (fig 4) In spite of the increase in heart rate during LBNP cardiac output was reduced by 33% in the unblocked condition due to a 60% decrease in stroke volume at the end of the exposure Propranolol augmented the decrease in cardiac output during LBNP even though the decrease in stroke volume was less pronounced than before blockade After combined blockade the percentage decrease in cardiac output was 46% the absolute value during LBNP however not being significantly different from that obtained with propranolol only Total peripheral resistance showed a prompt increase by 50% at the application of LBNP but then gradually decreased to a final level of 40% at the end of the exposure This phenomenon which was also observed after combined blockade was responsible for the gradual fall in blood pressure Absolute values of total peripheral resistance at the end of LBNP were 0 95 1 16 and 1 20 units before and after propranolol and combined propranolol and atropine respectively

DISCUSSION

A Methodological considerations

In the experiments with increased gravitational force the 3 G level was chosen since it has been found to produce profound circulatory changes yet being well tolerable to subjects having been made accustomed to the subjective sensations of sustained accelerative stress (Bjurstedt Hansson and Ström 1959 Rosenhamer 1967 Linnarsson and Rosenhamer 1968 Bjurstedt Rosenhamer and Wigertz 1968)

The adjustments of the circulation to an increase in the gravitational force are due largely to the effects of increased hydrostatic pressures below and decreased hydrostatic pressures above the hydrostatic indifference point (HIP) the HIP being defined as the point where the intravascular pressures stay constant when the direction or magnitude of the force of gravity is altered (cf Gauer and Thron 1965) In the sitting position the HIP for the venous as well as the arterial system is located approximately at the level of the diaphragm (cf Gauer and Zuidema 1961) Assuming a hydrostatic pressure difference between the HIP and the level of the carotid sinuses of approximately 20 mm Hg (fig 1) an abrupt threefold increase in the gravitational force will increase this pressure difference to 60 mm Hg i e the mean pressure in the carotid sinuses will initially be lowered by 40 mm Hg Likewise the hydrostatic pressure in

the horizontally oriented legs (fig 1) being 25-30 mm Hg higher than that prevailing at the level of the HIP during normal gravity will be increased to approximately 80 mm Hg during the 3 G runs

Evidence has been presented by Thron et al (1967) that with changes in carotid sinus transmural pressure accomplished by the technique employed in the present experiments the pressure transmitted to the walls of the carotid sinuses is linearly related to the applied helmet pressure when the latter is changed in the range -60 to +60 mm Hg Furthermore Eckberg (1976) found that maximum reduction of the internal jugular vein pressure was nearly equal to the reduction in pressure on the neck It can thus be assumed that in the present experiments the application of positive and negative pressures of 40 mm Hg in the helmet produced equal transmural pressure changes over the carotid sinuses in opposite directions from the normal

When the external pressure over the head and neck is decreased the driving pressure in the enclosed vascular segments approximates the difference between inflow and outflow pressures except initially for as long as it takes for the enclosed distensible veins to fill and the pressure in these vessels to approximate the outflow pressure so that flow is resumed Thus at steady state a subatmospheric helmet pressure does not affect the driving pressure in cerebral circuits However with positive pressure in the helmet the pressure in the downstream ends of the enclosed vascular segments is close to that of the surrounding pressure This

is due to the operation of the waterfall effect which characterizes the outflow from collapsible veins when the surrounding pressure is higher than the outflow pressure (cf Permutt and Riley 1963) This means that with a positive pressure in the helmet the transmitted portion of this pressure was lost from the normal driving pressure (inflow pressure minus outflow pressure) The functional implications of such loss of driving pressure have been considered in paper II (p 502)

The technique employed to produce lower body negative pressure has been used extensively during the last ten years (for review see Wolthuis Bergman and Nicogossian 1974) The application of LBNP causes an elevation in lower body vascular transmural pressure the magnitude of which is directly related to the level of reduced pressure applied (Coles 1956) thus producing pooling of blood in the distensible veins of the lower body and a consequent reduction in central blood volume To make possible a comparison between the results obtained in the LBNP experiments and those observed in the experiments with increased gravitational stress a level of negative pressure was chosen that would produce approximately the same increase in vascular transmural pressure in the legs as that obtaining during 3 G in the sitting position with the legs horizontally oriented viz 80 mm Hg (see above and fig 1) Since blood pooling during accelerative stress occurs primarily in the legs with little if any pooling in abdominal vessels (cf Howard 1965) it can be assumed that 80 mm Hg of LBNP will produce

approximately the same degree of blood pooling in the legs as will exposure to 3 G Such pooling reduces central blood volume and this is reflected by a proportional change in stroke volume (Sjöstrand 1953 Milnor Jose and McGaff 1960 Levinson Frank and Hellems 1964 Levinson Pacifico and Frank 1966 Ellis and Julius 1973) It is of interest that the values of stroke volume during 3 G and LBNP were almost identical (40 and 43 ml respectively) when the heart was deprived of autonomic influence This observation supports the assumption that the degree of peripheral pooling and reduction of central blood volume was approximately the same during the two experimental conditions

Since external temperature is known to strongly influence the amount of blood pooled (Henry 1951) care was taken to keep this variable constant during the experiment This was achieved by means of continuous ventilation of the LBNP-box during the control period as well as during the exposures to negative pressure Temperature could thus be kept within ±0 5°C in the box throughout the experiment

Beta-adrenergic and parasympathetic blockade of the heart A salient feature of the present investigation was to study the contribution of cardiac effector mechanisms to circulatory homeostasis in certain conditions of cardiovascular stress For this purpose beta-adrenergic and parasympathetic influence on cardiac activity was offset by the intravenous administration of propranolol and atropine separately and in combination

Epstein et al (1965) found that 0 15 mg/kg of propranolol

intravenously produced a degree of blockade that caused at least a tenfold reduction in the sensitivity of the heart to infused isoproterenol Jose and Taylor (1969) found no further decrease in resting heart rate when increasing the dose from 0 20 to 0 25 mg/kg However the latter dose would seem preferable in conditions with a high sympathetic background activity such as presumably existed in the present experiments on cardiovascular effects of increased gravitational stress with and without exercise and of LBNP Robinson et al (1966) who investigated the effects of exercise and postural changes on the heart rate used a dose of 0 25 mg/kg This dose has been used throughout in the present experiments Unlike certain other beta-adrenergic blocking agents propranolol is devoid of any intrinsic sympathomimetic activity in the heart (for review see Gibson 1974)

The primary actions of atropine on the heart are blockade of vagal negative chronotropic and inotropic effects (for review see Higgins Vatner and Braunwald 1973) However apart from its cardiac vagal blocking effects atropine has several other sites of action (see further below) which may interfere with the cardiovascular reflexes Since these effects of atropine may affect tolerance to stressful stimuli such as those employed in the present experiments a dose as small as possible to produce effective cardiac vagal blockade was chosen (0 03 mg/kg) The dose required to produce maximum inhibition of vagal control of the sinoatrial node has been reported to be in the range 0 025 to 0 04 mg/kg (Chamberlain Turner and Sneddon 1967) Jose and Taylor (1969) found that

increasing the dose from 0 03 to 0 04 mg/kg raised the resting heart rate by only 0 8 beats/min In pilot LBNP experiments (8 subjects unpublished observations) preceding the present investigation a substantial slowing of the heart was noted after release of LBNP during cardiac effector blockade Since it could not be excluded that this effect on the heart was due to defective vagal blockade the dose of atropine was increased to 0 04 mg/kg in the present LBNP experiments It was found however that the increased dose had no significant influence on the post-LBNP slowing of the heart rate

It is well known that both propranolol and atropine exerts other influences on the circulation than those resulting from blockade of cardiac effector mechanisms Thus propranolol as well as atropine may interfere with the circulatory reflex control by prevention of beta-adrenergic and cholinergic vasodilatation respectively even though the significance of such vasodilatation in the baroreflex control of vascular tone in man i uncertain (Brick 1966 Mahon 1966 Johnsson 1967 Price Cooperman and Warden 1967 Folkow 1955 Roddie and Shepherd 1963, Uvnäs 1967) Furthermore atropine may affect certain cardiovascular reflex responses due to its central ganglionic and peripheral sites of action (for reviews see Ambache 1955 Eger 1969) The importance of these effects of propranolol and atropine will be discussed where relevant in later sections

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lume by loss of baroreceptor-induced inotropic drive to the heart (Sarnoff et al 1960 De Geest Levy and Zieske 1964 Glick 1971); it has been shown that exercise-induced augmentation of the contractile state of the myocardium is diminished by propranolol (Sonnenblick et al 1965) and that beta-adrenergic blockade diminishes cardiac output also when heart rate is kept constant by fixed rate artificial pacemakers (Bloomfield and Sowton 1967 Donoso et al 1967 Lund--Larsen et al 1968) Since in the present experiment on circulatory adjustments to a change from the supine to the erect posture the exaggerated curtailment of cardiac output after blockade was well compensated for by a concomitant increase in vascular resistance it is concluded that cardiac effector mechanisms are not of essential importance for the maintenance of arterial pressure during orthostasis This conclusion is in agreement with the results of several investigations concerning orthostatic tolerance in man during beta-adrenergic blockade (Sannerstedt Julius and Conway 1970 Cuthbert and Owuso-Ankomah 1971 Fuccella et al 1971 Maksud et al 1971 Spodick Meyer and St Pierre 1972)

When the gravitational force acting in the long axis of the body is further increased (I III) the circulatory responses are not solely exaggerated but in part qualitatively different from those obtained by head up tilt Thus at 3 G there is a more pronounced increase in heart rate (Bjurstedt Hansson and Ström 1959) concomitant with a decrease in stroke volume which leaves cardiac output only slightly or moderately depressed (Lindberg et al 1969 Rosenhamer 1967) Due to a

potent vasoconstrictor response mean arterial pressure at heart level is not only maintained but is actuallay increased sometimes quite markedly (Lindberg et al 1960) These findings are confirmed by the results obtained in the present experiments (I III)

The fact that the circulatory responses to increased gravitational force before blockade were almost identical in studies I and III makes a comparison between values obtained after beta-adrenergic and combined cardiac blockade respectively in the separate studies justifiable although the experiments were performed on different days and with only some of the subjects participating in both studies After propranolol alone the response of the heart rate to 3 G was only 38% of that obtained without this drug (fig 4) The impaired heart-rate response was accompanied by a more clearcut curtailment of cardiac output than occurred before blockade In spite of this mean arterial pressure at heart level rose to about the same level before and after propranolol due to a more marked vasoconstrictor response after blockade (fig 3) The fact that the G-induced vasoconstrictor response was so precisely adjusted to compensate for the impaired response of the heart indicates not only that the central regulation of the arterial pressure was intact after propranolol; it also implies that cardiac sympathetic drive is not an essential mechanism in the circulatory defense against increased gravitational stress This concept is further emphasized by the observations of the G-induced arterial pressure response during exercise; this response was not affected by proprano-

lol even though the heart rate response to increased gravitational force during exercise was completely abolished by this drug (fig 3 and 4)

After combined cardiac effector blockade the G-induced increase in mean arterial pressure at heart level was significantly reduced (fig 3) Since propranolol alone did not affect the blood pressure response the explanation for the defective regulation of arterial pressure at 3 G after combined blockade must be sought in possible deleterious actions of atropine alone or in combination with propranolol on cardiac output or total peripheral resistance Since the mechanism of action of atropine on the heart is blockade of vagal negative chronotropic and inotropic effects it appears less probable that any reduction in cardiac performance at 3 G after combined heart blockade was due to actions of atropine on the heart On the other hand it has been suggested that atropine may impair cardiac filling in ortho tasis due to increased blood pooling in infracardiac vessels (Kalser Frye and Gordon 1954) Furthermore other possible actions of atropine such as interference with some of the actions of acetylcholine on various parts of the central nervous system or a partial ganglionic blockade (for reviews see Ambache 1955 Eger 1962) may have acted to prevent the baroreceptor-induced vasoconstrictor response from fully developing as required for the maintenance of arterial pressure homeostasis

One of the salient effects of increased gravitational force in the head-seat direction is a fall of the mean arterial pressure in the cranial portion of the body It was

therefore thought of interest to study certain circulatory responses to primary pressure changes at the level of the carotid sinuses (II) Such changes were produced by altering the pressure in a helmet enclosing the head and neck It was found that a decrease in carotid sinus transmural pressure produced a much stronger arterial pressure response than a corresponding increase in transmural pressure (fig 3) The finding that the carotid baroreflex acts as a predominantly antiphypotensive mechanism is in agreement with the results of Thron <u>et al</u> (1967) and Stegemann Busert and Brock (1974) who used a similar technique but contrasts with the generally held concept that the stimulus-response relationship of the carotid sinus reflex is a sigmoid curve which is symmetrical about normal arterial pressure (<u>cf</u> Heymans and Neil 1952) However this concept has arisen from findings in anesthetized vagotomized animals with surgically exposed and isolated open loop carotid sinus preparations; it is generally agreed that anesthesia as well as surgical trauma may interfere with the baroreflex response characteristics (<u>cf</u> Vatner and Braunwald 1975 Kirchheim 1976)

Concomitantly with the stronger pressure response to decreased transmural pressure cardiac output showed a significant 10% increase whereas no change in this variable was observed when the transmural pressure was increased Thus the difference between the magnitudes of the blood pressure re sponses in the two cases was associated with a change in the relative contributions of cardiac and peripheral effector mechanisms After combined cardiac effector when

any intervention on the part of the cardiac effector mechanisms was prevented the difference in magnitude between the blood pressure responses to increased and decreased transmural pressure was somewhat less evident However the fact that the reflex blood pressure responses to changes in transmural sinus pressure were not greatly impaired in the absence of cardiac effector mechanisms indicates that these mechanisms are not of critical importance in the buffer function of the carotid sinus baroreceptors (fig 3) This conclusion is in agreement with the results of Ernsting and Parry (1967) who increased the carotid sinus transmural pressure by suction in a box enclosing the neck only It is also concordant with the observation of Epstein <u>et al</u> (1969) that electrical stimulation of the carotid sinus nerve in man causes a fall in systemic arterial pressure even when cardiac output is kept constant my mild exercise and with that of Eckberg <u>et al</u> (1972) that the arterial pressure response to carotid sinus nerve stimulation is not affected by autonomic heart blockade

Besides its lowering effect on the arterial pressure at carotid sinus level gravitational stress in the head-seat direction produces a downward displacement of blood volume from the intrathoracic space to infracardiac veins The reflex circulatory responses to a reduction in central blood volume without an accompanying fall in mean arterial pressure may be studied by lowering the external pressure over the lower part of the body In the supine body position th maneuver may produce considerable pooling of blood in depen-

dent veins with little if any effect on the arterial pressure at head level (for review see Wolthuis Bergman and Nicogossian 1974) In the present experiments a level of negative pressure (80 mm Hg) was chosen that produces blood pooling of an order of magnitude comparable to that produced in the 3 G experiments It was of interest to note that 80 mm Hg of LBNP produced a more clearcut reduction in cardiac output and a less pronounced vasoconstrictor response than did exposure to 3 G (fig 2)

After propranolol tolerance to LBNP was reduced as judged from the blood pressure response i e mean arterial pressure was significantly lower at the end of the LBNP period than it was before blockade (fig 3) This difference can be explained by the blockade of cardiac sympathetic effector mechanisms and a resulting reduction in cardiac output Although there was a more potent vasoconstrictor response to LBNP after propranolol it was not sufficient to maintain arterial pressure at the same level as before blockade This finding is notable since the exaggerated curtailment of cardiac output following exposure to 3 G after propranolol was well compensated by a concomitant rise in peripheral resistance (I) It is believed therefore that the carotid hypotension associated with exposure to increased gravitational force initiated a stronger overall sympathetic outflow than did exposure to LBNP

With combined cardiac effector blockade heart rate cardiac output and mean arterial pressure increased considerably these findings being concordant with those of other experiments concerning autonomic heart blockae in the re-

cumbent position (Cummings and Carr 1966 Julius et al 1971 Ellis and Julius 1973 Korner et al 1973 Korner Tonkin and Uther 1976) However following application of LBNP there was a drastic reduction in cardiac output and mean arterial pressure to the same levels as obtained during LBNP with propranolol only although the heart rate remained elevated (fig 3 and 4) These findings clearly show the dependence of the central blood volume for the capacity of the heart to increase its output by means of an increase in its rate and are in agreement with earlier results concerning atropine and LBNP (Murray and Shropshire 1970) and atropine and posture (Weissler Leonard and Warren 1957) It is thus concluded from the LBNP experiments that cardiac sympathetic effector mechanisms were essential for the maintenance of arterial pressure

In summary the present experiments on the effects of head-up tilt increased gravitational force and locally lowered carotid sinus transmural pressure have shown that cardiac effector mechanisms are not of essential importance for the reflex blood pressure regulation associated with such maneuvers In all these cases carotid sinus transmural pressure was markedly reduced On the other hand cardiac sympathetic drive may be essential for the circulatory homoeostasis in conditions with reduced central blood volume such as LBNP when mean arterial pressure at carotid sinus level is only slightly affected

Effects of posture and exercise on baroreceptor-induced changes in heart rate and mean arterial pressure

There is still considerable controversy as to what extent postural changes and exercise may modify baroreflex control of heart rate and arterial pressure When in the present experiments the carotid sinus transmural pressure was increased or decreased by negative and positive pressure on the neck respectively the ensuing heart-rate responses were markedly altered by a change in posture Thus during head-up tilt the heart-rate response was only half that obtained in the supine position This is in accordance with the findings of Pickering et al (1971) that the reflex bradycardia in response to a drug-induced rise of arterial pressure is decreased in the erect posture but is at variance with those of Eckberg Abboud and Mark (1976) who reported that the decrease in heart rate evoked by negative pressure on the neck is the same in the supine and standing positions In the latter investigation however observations were limited to the first 5 sec of suction on the neck in order to minimize cardioacceleration resulting from reduction of aortic baroreceptor activity by reflex hypotension In the present experiments on the other hand measurements were obtained after 30 sec of negative pressure i e when the baroreceptor-induced reduction in mean arterial pressure was fully developed The discrepancy between the results obtained in the two studies may thus be explained by different contributions on the part of the aortic baroreflex on the heart-rate responses

In the present experiments it was observed that exercise reduced the G-induced cardioacceleration by nearly 50% (fig 4) Analogue results were obtained by Pickering et al (1972a) in their studies on the effects of exercise on the transient increase in heart rate in response to a drug-induced decrease in arterial pressure while Robinson et al (1966) found that the changes in heart rate that followed sustained alterations in arterial pressure were of similar magnitudes at rest and during exercise However whereas the changes in heart rate studied by Robinson et al (1966) and Pickering et al (1972a) were induced by alterations in arterial pressure the G-induced acceleration of the heart is the result of central hypovolemia as well as carotid hypotension A comparison between results obtained by the two methods is therefore only of limited relevance

The above observed effects of posture and exercise on the heart-rate responses were not reflected in corresponding alterations in the baroreceptor-mediated changes in arterial pressure Thus the pressor and depressor responses to positive and negative pressure on the neck respectively were as potent in the supine as in the 70° head-up position Furthermore the G-induced increase in mean arterial pressure was almost exactly the same before and during leg exercise (fig 3) It can be concluded therefore that the carotid baroreflex responsiveness in terms of its buffering capacity of acute changes in arterial pressure is not modified by exercise or postural shifts even though the contribution on the part of the heart rate in the reflex response may be attenuated by

maneuvers These findings give further support to the conclusion drawn in the previous section viz that cardiac effector mechanisms are not of essential importance for the reflex arterial pressure regulation associated with changes in the carotid sinus transmural pressure or exposure to increased gravitational force

It was found in the present experiments that the pressor response to a threefold increase in the gravitational force was much more potent than that following positive pressure on the neck although the decrease in carotid sinus transmural pressure was essentially the same in the two situations (fig 3) Several explanations to this disbrepancy must be considered A more potent G-induced pressor response might be expected from stronger ischemic stimulation of carotid chemoreceptors due to local reduction of perfusion pressure (cf Landgren and Neil 1951) This seems less probable however since the perfusion pressure at the level of the carotid bodies was essentially the same in the two situations Furthermore there is no decrease in arterial oxygen tension or increase in arterial carbon dioxide tension during exposure to 3 G (Rosenhamer 1967) The possibility must therefore be considered that the external pressure applied to the neck in study II was not completely transmitted to the walls of the carotid sinuses However if this were to explain the entire difference in magnitude between the pressor response to 3 G and that of positive pressure on the neck respectively the external pressure must have been greatly attenuated during the transmission through the tissues of the neck to

the walls of the carotid sinuses This appears less probable since Eckberg (1976) found that maximum reduction of the internal jugular vein pressure is nearly equal to maximum reduction in neck chamber pressure

The validity of the comparison between exposure to 3 G and 40 mm Hg of neck pressure is based on the assumption that the intrasinus mean pressure was lowered by 40 mm Hg by the transition from 1 G to 3 G (cf fig 1) However if this pressure reduction was in reality greater the pressor response to 3 G would presumably be more potent than that obtained by 40 mm Hg of pressure on the neck Finally changes in the carotid sinus transmural pressure produced by alterations of the external pressure on the neck will only affect the static component of the carotid baroreflex Exposure to 3 G on the other hand also alters the dynamic component due to drainage of blood from the intrathoracic space and an ensuing decrease in pulse pressure It is well known that alterations in intrasinus pulse pressure constitute potent stimuli to the carotid inus baroreceptors (Ead Greer and Neil 1952 Scher and Young 1963 Gero and Gerova 1967 Angell James and Daly 1970); this may therefore form an explanation why the pressor response to 3 G was more potent than that during 40 mm Hg or neck pressure

Relative contribution of sympathetic and parasympathetic influence in reflex heart-rate control

Although there ha been considerable interest in the mechanism by which the heart rate is altered in response to

postural changes and stimulation of the baroreceptors the question concerning the relative roles of the two divisions of the autonomic nervous system in mediating these changes in conscious man has not yet been settled When subjects were exposed to 3 G in the seated position on the centrifuge it was observed that propranolol reduced the normal response of the heart rate to increased gravitational force by 62% (fig 4) Thus G-induced cardioacceleration is predominantly but not entirely due to sympathetic stimulation The fact that the addition of atropine raised heart rate considerably at normal gravity but only slightly at 3 G indicates that there was a considerable vagal restraint at 1 G which was almost completely withdrawn at 3 G (fig 4) When exercise was performed during exposure to 3 G the G-induced heart-rate response was almost entirely of sympathetic origin since propranolol reduced this response by no less than 92% During exposure to LBNP propranolol reduced the reflex heart-rate response by 51% However the addition of atropine increased heart rate considerably not only before but also during LBNP indicating the existence of strong vagal restraint even during LBNP (fig 4) It can thus be concluded that the reflex heart-rate response to a reduction in central blood volume separately or in combination with an unloading of arterial baroreceptors is of both parasympathetic and sympathetic origin the ultimate balance however being dependent on the preexisting level of background activity The observations of a reciprocal control of the heart rate is in agreement with the findings of Glick and Braunwald (1965)

Robinson et al (1966) and Scher et al (1970) but are at variance with those of Leon Shaver and Leonard (1970) and Pickering et al (1972a) who claim that baroreceptor-induced changes in heart rate are entirely of parasympathetic origin The last-mentioned groups studied transient effects on heart rate whereas the present results refer to effects studied over several minutes It is well known that parasympathetic control of the heart rate is extremely fast sympathetic control being considerably slower (Wang and Borison 1947 Warner and Cox 1962); thus the relative contributions of the two divisions of the autonomic system may not be the same for transient as for the sustained heart-rate responses

The finding that the heart-rate responses to 3 G and LBNP were of similar magnitude whereas those following isolated changes in carotid sinus transmural pressure were much less conspicuous (fig 4) suggests that the main component responsible for the orthostatic heart-rate control is the decrease in central blood volume the lowering of intrasinus mean pressure being of less importance On the other hand intrasinus mean pressure eems to be an important factor for the vasocon trictor response to orthostasis since the increase in total peripheral resistance was much more conspicuous following head up tilt and 3 G as compared to LBNP (see above and fig 2)

SUMMARY

The objectives of the present investigation were to study in conscious man some of the functions of cardiovascular reflex mechanisms in certain experimental conditions *viz* 70° head-up tilt (II) a threefold increase of the force of gravity (I III) bidirectional changes (± 40 mm Hg) in the pressure surrounding the neck in the supine and 70° head-up position (II) and lower body negative pressure (LBNP 80 mm Hg) (IV) In this way it was possible to study different circulatory effects primarily induced by changes in the transmural pressure at the level of the carotid sinuses and by reductions in central blood volume separately or in combination

Intraarterial pressure electronically derived mean arterial pressure heart rate and the actual G-level or the external pressure applied to the neck or the lower part of the body were recorded continuously and simultaneously Cardiac output determinations were obtained by the dye-dilution technique Stroke volume and total peripheral resistance were calculated from simultaneously obtained values of cardiac output and heart rate or mean arterial pressure respectively

In order to further elucidate the contributions of cardiac effector mechanisms to the reflex effects on the circulation the above-mentioned circulatory variables were observed before and after beta-adrenergic and/or combined beta-adrenergic and parasympathetic blockade as induced by the i v administration

of propranolol separately or in combination with atropine

After combined cardiac effector blockade a shift from the supine to the 70° head-up position caused a greater fall in cardiac output than before blockade However mean arterial pressure at heart level was not lowered and the exaggerated curtailment of cardiac output was therefore fully compensated by a greater increase in systemic vascular resistance

The response of the heart rate to increased gravitational force was markedly reduced by propranolol The impaired heart-rate response was accompanied by a more clearcut curtailment of cardiac output than occurred before blockade In spite of this mean arterial pressure at heart level rose to the same level as before propranolol due to a more marked vasoconstrictor response after blockade Combined cardiac effector blockade reduced the G induced arterial pressure response This effect is discussed in terms of actions of atropine on the baroreceptor-induced vasoconstrictor response and/or on the degree of infracardiac pooling

A decrease in carotid sinus transmural pressure produced a much stronger arterial pressure response than a correspond ng increase in transmural pres ure Th s ind cates that n man the carotid sinus reflex acts as a predominantly antihypotensive mechanism Whereas the depressor respon e to increa ed transmural pressure was due solely to a reduct on in vascular resistance an increase in cardiac output contributed to the mor potent pre sor response to decreased transmural pressu e The pressor nd depressor responses to changes in the transmural pressure were a potent in th supine as n the 70° head-

-up position Combined cardiac effector blockade abolished t effect of reduced transmural pressure on cardiac output without impairing the blood pressure response

Tolerance to LBNP was reduced by beta-adrenergic blockade as judged from the exaggerated decrease in mean arterial during LBNP after blockade Combined cardiac effector blockade elevated pre-LBNP values of heart rate cardiac output and arterial pressure as compared to values obtained after beta--adrenergic blockade alone; however tolerance to LBNP rem ' impaired

From the experiments on the effects of head-up tilt increased gravitational force and locally lowered carotid si transmural pressure it is concluded that cardiac effector mechanisms were not of essential importance for the reflex blood pressure regulation associated with these maneuvers In all these cases carotid sinus transmural pressure was markedly reduced However during LBNP when mean arterial pressure at carotid sinus level is only slightly affected but the central blood volume is effectively reduced cardiac sympathetic drive seemed to be important for the maintenance of arterial pressure

Experiments with beta-adrenergic and combined autonomic heart blockade showed that G-induced cardioacceleration was predominantly but not entirely due to sympathetic stimulation When exercise was performed during exposure to 3 G the G in heart-rate response was almost entirely of sympathetic origin There was a considerable vagal restraint at 1 G at rest which was almost completely withdrawn at 3 G The heart-rate response to LBNP was equally attributable to withdrwal of vagal tone and

increase in sympathetic drive a considerable vagal restraint still remaining in this condition From these findings it is concluded that the reflex heart-rate response to a reduction in central blood volume separately or in combination with an unloading of arterial baroreceptors is of both parasympathetic and sympathetic origin the ultimate balance being dependent on the preexisting level of background activity

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ACTA PHYSIOLOGICA SCANDINAVICA SUPPLEMENTUM 447

PLASMA CHOLINE AND CHOLINERGIC MECHANISMS IN THE BRAIN

METHODS FUNCTION AND RÔLE IN HUNTINGTON S CHOREA

BY
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UPPSALA 1977

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Preface

About six blind men of Indostan who approached an elephant for the first time·

First blind man falling against the elephant s side
"God bless me! but this elephant
Is very lika a wall"
Second blind man feeling the tusk
"This wonder of an elephant
Is very like a spear"
Third blind man grasping the squirming trunk
I see quoth he "the elephant
Is very like a snake
The fourth blind man clasping the knee
"This is clear enough the elephant
Is very like a tree
The fifth blind man catching the ear
"This marvel of an elephant
Is very like a fan
The sixth seLing the swinging tail.
I see quoth he the elephant
Is very like a rope
And so these men of Indostan
Disputed loud and long
Each in his own opinion
Exceedingly stiff and strong
Though each was partly in the right
And all were in the wrong

(William Shenstone 1714–1763)

1 A Short History of Cholinergic Biology

The origin of cholinergic biology can be traced back to the experiments by Hunt and Taveau (1906), in which the physiological effects of different choline analogues were examined. They observed that the most potent vasodepressor analogue was the acetyl derivative acetylcholine (ACh).

In 1914 Sir Henry Dale suggested that ACh was a substance of importance for the function of the peripheral nervous system and in 1929 were Dale and Dudley able to isolate ACh from mammalian tissue.

A pupil of Dale, Dikshit, was to my knowledge the first to suggest, on experimental grounds, that ACh had a function as a transmitter substance in the central nervous system (CNS) also (Dikshit 1934). However convincing data were sparse for a long time, but it became more and more generally accepted that ACh was in fact a transmitter in the CNS (Feldberg 1945).

The final identification of ACh in mammalian brain had to await the application of gas chromatography in combination with mass spectrometry (Hammar et al. 1968).

During the last few years there has been a rapid acceleration in the development of new methods for Ch and ACh determination, as well as for sacrifice of animals, turnover calculations, mapping of cholinergic structures within the brain, subcellular fractionating, determination of ACh release, sterotaxic intracerebral application of drugs, and neurophysiological methods for intracerebral stimulation and recording. In combination with neuropharmacological experiments these advances have almost dramatically increased our knowledge of the importance of the central cholinergic mechanisms.

2 Determination of Ch and ACh

2.1 Bioassay

Up to the end of the 1960's bioassay was the only method available for determination of ACh in the small quantities present in most tissues and tissue perfusates. Some of these assays are very sensitive, but the selectivity is always questionable to some degree since it is well known that other endogenous substances (for example serotonin and histamine) will also result in a biological response. In pharmacological experiments drugs will be present, mostly in unknown concentrations, in the test solutions and may increase or decrease the biological response (Hanin and Jenden 1966). Only when the specificity is carefully controlled can bioassay be used with success.

2.2 Radioisotopic Procedures

All radioisotopic methods for determination of Ch and ACh are based on the enzymatic conversion of Ch to a radioactive product whose activity is measured after isolation. The methods began with the development of sensitive radioenzymatic techniques for Ch determination (Smith and Saelens 1967, Schuberth et al. 1969, Ewetz et al. 1969) and these principles were originally applied to ACh measurement by Feigenson and Saelens (1969). The different steps in existing radioenzymatic methods are summarized below:

A *Separation of Ch and ACh*
a) Electrophoretically (Feigenson and Saelens 1969)
b) Enzymatic conversion of Ch to phosphorylcholine (Goldberg and McCaman 1973, Shea and Aprison 1973)
c) Separation not needed (Massarelli et al. 1976, Paper III).

B *Hydrolysis of ACh*
a) Base (Feigenson & Saelens 1969, Massarelli et al. 1976).
b) Acetylcholinesterase (AChE, Goldberg & McCaman 1973, Paper III).

C *Conversion of Ch to radioactive product*
a) Choline acetyltransferase (ChAT): Ch $\xrightarrow[\text{ChAT}]{\text{labelled Acetyl CoA}}$ labelled ACh (Feigenson & Saelens 1969, Papers II and III, Massarelli et al. 1976).
b) Choline phosphokinase: Ch $\xrightarrow[\text{choline phosphokinase}]{\text{ATP}}$ ^{32}P phosphorylcholine (Ewetz et al. 1969, Reid et al. 1971).

D *Separation of labelled precursor from product*
a) Electrophoretically (Feigenson & Saelens 1969).
b) Liquid cation-exchange chromatography (Shea & Aprison 1973)
c) Column anion-exchange chromatography (Reid et al. 1971).
d) Ion-pair extraction, with K_2HgI_4 in heptanon-(3) (Paper II)· with tetraphenylboron (Fonnum 1969) in butenenitrile (Shea & Aprison 1973) with tetraphenylboron in acetonitrile/toluene (Fonnum 1975 Paper III)· with tetraphenylboron in diisobutylketone (Massarelli et al. 1976).

2.3 Gas Chromatographic Procedures

Several gas chromatographic procedures for Ch and ACh determination have been described (Jenden et al. 1968 Hammar et al. 1968 Hanin et al. 1970· Hanin & Skinner 1975 Szilagy et al. 1972). The most specific of all methods for assaying Ch and ACh is probably the application of gas chromatography in combination with mass spectrometry (Hammar et al. 1968 Hanin & Skinner 1975), but in view of the extreme cost of such equipment it cannot be expected to come into use as a routine instrument in most laboratories.

2.4 Other Methodological Approaches

Several other methods for Ch and ACh determination have been described (see Hanin 1974) for example: a) Polarographic (Maslova 1964) b) Photometric (Schumacher & Ehl 1970· Eksborg & Persson 1971a,b c) Fluorometric (Fellman 1969) d) Ion pair partition chromatography (Ulin et al. 1976). None of these methods have yet been of any great importance for the recent development of cholinergic biology

2.5 Methods Used in Present Studies

2.5.1 Bioassay of ACh

In study I a strip of the dorsal muscle of the leech was used in a microbath (Szerb 1961) for the determination of ACh. As pointed out earlier the specificity of the analytical method had to be checked in several ways
a) When known amounts of ACh were added to the tissue extracts no potentiation or inhibition occurred.
b) Extracts treated with alkali (pH 11–12) had no biological effect.
c) Addition of drugs (haloperidol and apomorphine) to extracts did not result in any potentiation or inhibition of the biological response to ACh.
d) Some aliquots of brain extracts analysed by gas chromatography/mass spectrometry gave values similar to those obtained for bioassayed aliquots.

The bioassay procedure had a very low capacity however, and further the

progress of our work seemed to be more and more dependent upon a good leech season in Hungary. This inspired us to develop a radioenzymatic method for Ch and ACh assay (Papers II and III see below).

2.5.1.1 Results and discussion. As the experiments in study I were performed before the introduction of microwave irradiation for the stabilization of Ch and ACh levels, the endogenous values of ACh are considerably lower than those obtained in subsequent studies, in which the animals were killed by microwave irradiation (Paper III see below). This is probably due to rapid postmortal hydrolysis of an AChE-sensitive ACh pool (see Nordberg 1977). The levels of endogenous ACh obtained in study I are of the same magnitude (cortex 10 nmol g^{-1} striatum 29 nmol g^{-1}) as those reported earlier by other authors using decapitation or near-freezing methods to kill the animals (for review of all ACh concentrations in rat and mouse brains published earlier see Saelens and Simke 1976).

2.5.2 Determination of free Ch in plasma (Paper II)

In study II a simple and rapid assay system for plasma Ch based on radioenzymatic determination in an ultrafiltrate was applied in an investigation on healthy volunteers.

2.5.2.1 Ultrafiltration of plasma. The concentration of free Ch increased, probably due to an enzymatic process, when plasma and blood were left at room temperature. In plasma and blood kept at +4°C and in a protein-free ultrafiltrate of plasma kept at 22°C no increase in Ch concentration was detected (Fig. 1). Thus, rapid cooling of blood after venepuncture, followed by ultrafiltration of the plasma, seemed to be a good way to obtain a stable plasma sample for further processing. An increase in Ch concentration has also been found to occur in plasma from experimental animals (Wang and Haubrich 1975 Spanner et al. 1976). Spanner et al. (1976) used formic acid/acetone extraction of the precooled plasma sample to obtain a stable value. This method seems to be useful for that purpose, but it is very time-consuming. However the plasma concentrations obtained in their study (Spanner et al. 1976) are extremely low (~0.8 nmol ml^{-1} in human plasma). The reason for this seems to be unexplainable at present. Wang and Haubrich (1975) boiled plasma for 15 min in an attempt to get stable Ch values. This method is rapid, but as shown in paper II it seems to result in significantly higher values than those obtained with the ultrafiltration procedure probably due to a rapid initial release of Ch from Ch-containing compounds. Ultrafiltration of plasma, on the other hand, separates low molecular weight Ch containing compounds from the enzymes catalyzing the Ch liberation without any increase in temperature or changes in other physical properties. The concentration of Ch in the ultrafiltrate probably corresponds to free Ch of plasma, which equilibrates physiologically with other Ch compartments in the body.

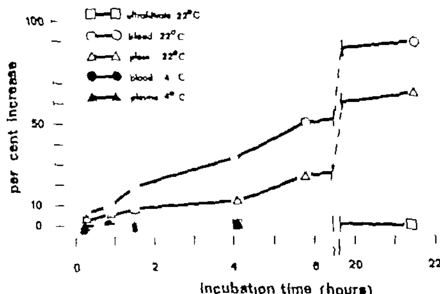


Fig. 1 Choline concentration in ultrafiltrate, blood and plasma left for different times at room temperature (22°C) and at 4°C. The curves of ultrafiltrate (22°C), blood and plasma at 4°C are superimposed in the time interval 0–4 (from Paper II).

Table I Choline concentration in ultrafiltrate of human plasma (µmol/l mean ± S.E.M.)

Group	8 a.m. fasted 10–14 hrs	Confidence limits 95 %	1 p.m. (one hr after meal)	Confidence limits 95 %
Children (4–11 years, 3♂, 5♀)	9.7 ± 0.6 n = 8	8.3 – 11.1	xxx 12.1 ± 0.3 n = 8	11.4 – 12.8
Adults (24–57 years, 5♂, 5♀)	9.7 ± 0.6 n = 10	8.3 – 10.9	xx 11.0 ± 0.5 n = 10	9.7 – 12.2
Adults (70–74 years, 3♂, 2♀)	12.6 ± 0.3 n = 5	11.7 – 13.6	11.5 ± 0.9 n = 5	9.1 – 13.9
Mean 11♂, 12♀	10.6 ± 0.4 n = 23	9.6 – 11.4	xx 11.5 ± 0.3 23	10.8 – 12.2

Significantly different (xx $p < 0.01$ x $p < 0.001$ Student's paired t-test) from fasted Ch value (8 a.m.)

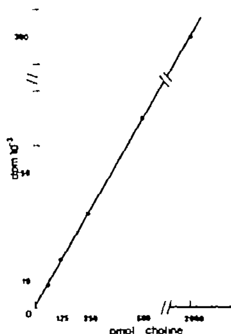


Fig. 2 A typical standard curve used in Ch determination. Each point represents the mean of two determinations. S.E.M at each point is less than 2 % of the mean r = 0.99 k = 1500 ± 2 % (± S.D) (from paper III).

2.5.2.2 Results and discussion. Using two determinations at five different Ch concentrations as standards, the detection limit of Ch in a typical standard curve (fig. 2) at a 95 % confidence level (Hubaux and Vos 1970) is about 25 pmol.

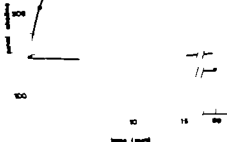
The plasma Ch concentrations in healthy fasting volunteers lay within narrow limits (mean 10.6 µmol/l). A small but significant increase was noted one hour after a meal (11.5 µmol l) (Table I). The concentration range was similar to that reported in an early investigation by Bligh (1952), who used a bioassay technique for Ch determination.

In three newborn infants the free Ch concentration in plasma (obtained from the umbilical vein) was about double (24.5 µmol l) that in older children and adults. No explanation can be given for this as yet but it is known that the placenta is extremely rich in ACh and the cholinergic enzymes ChAT and AChE (Sastry et al. 1976).

2.5.3 Determination of Ch and ACh in rat brain parts (Paper III)

This paper describes a modification of the previously used radioenzymatic procedure for Ch and ACh determination (Feigenson and Saelens 1969 Goldberg and McCaman 1973 Shea and Aprison 1973). After ion pair extraction of the ^{1}C ACh formed, using tetraphenylboron (Kalignost®), directly in the scintillation vial (Fonnum 1975), radioactivity is measured by liquid scintillation counting. In one aliquot of a brain extract endogenous ACh is specifically hydrolyzed by

Fig. 3 Free Ch following incubation with acetylcholine esterase (AChE) for different times at 37° C, pH=8. ●—● striatal homogenate; ▼—▼ a solution containing 2000 pmol phosphorylcholine and 200 pmol Ch □—□ striatal homogenate with no AChE added (from paper III).



means of AChE (Fig. 3) and the total Ch content of the sample is determined. The content of endogenous Ch is measured in an unhydrolyzed aliquot and the ACh level is then obtained as the difference in Ch content between the two aliquots.

2.5.3.1 Microwave irradiation for stabilizing Ch and ACh levels Since the Ch and ACh concentrations in brain change extremely rapidly *post mortem* (Ewetz et al. 1969, Schuberth et al. 1970, Dross and Kewitz 1972, Nordberg 1977a), it is necessary to use a technique which quickly inactivates the enzymes responsible for these changes. This can be done by rapid heating of the brain by microwave irradiation (Stavinoha et al. 1973, Butcher and Butcher 1974a, Guidotti et al. 1974, Schmidt 1976, Nordberg and Sundwall 1976, Nordberg 1977a). It has been shown that a temperature of 80–85°C is sufficient to inactivate brain AChE and ChAT and thereby eliminate *post mortem* alterations in ACh steady state levels (Schmidt et al. 1972, Schmidt 1976, Stavinoha et al. 1974, Nordberg and Sundwall 1976, Nordberg 1977). However, as shown by Schmidt (1976) a brain temperature of about 95°C must be attained in order to halt the post mortem increase in Ch concentration in rat brain. This indicates that the enzyme(s) responsible for producing post-mortem increases in Ch are more heat stable than both AChE and ChAT. In studies III and IV a beam of microwave (5kW, 2450 MHz) was focused on the rats' heads for 1.7 s. This resulted in a homogenous brain temperature of about 95°C. However, the possibility cannot be ruled out that changes still take place during the irradiation period or during the time while the animals are being restrained immediately before death. A "stress-free" method of killing cannot be achieved at present (if ever).

2.5.3.2 Results and discussion The radioenzymatic method described in Paper III was found to be a rapid (120 samples/day) and simple procedure for the measurement of Ch and ACh concentrations in small parts of brain tissue. The detection limit of a typical standard curve at a 95 % confidence level (Hubaux

Table II *Recently published values for Ch and ACh concentration in rats and mice sacrificed by microwave irradiation*

Analytical procedure	Authors	Cortex Ch	Cortex ACh	Striatum Ch	Striatum ACh
		nmol/g ± S.E.M.			
Radioenzymatic	Present paper	23±2.5 n = 18	16±1.0 n = 18	33±1.2 n = 23	76±2.4 n = 23
	Hrdbrich et al. 1975	17	14	29	83
Gas chromatography	Weintraub et al. 1976	–	16	–	64
	Racagni et al. 1976	–	–	39	51
	Modak et al. 1976*	–	26	–	81
	Schmidt 1976	28	28	32	64
Gas chromatography/ mass spectrometry	Butcher and Butcher 1974	–	–	27	98
Bioassay	Nordberg and Sundwall 1977*	31	24	41	74

*Mouse

and Vos 1970) is about 25 pmol (Fig. 2). The calculation of ACh concentration as the difference in Ch concentration between a hydrolyzed and an unhydrolyzed aliquot obviates the separation of Ch from ACh by chromatography or electrophoresis, which are time-consuming procedures that sometimes do not give complete separation of the two compounds. The extraction procedure for ACh described by Fonnum (1975), where ^{14}C ACh is separated from ^{14}C-acetyl-CoA directly in the scintillation vials prior to liquid scintillation counting, was found to be very convenient and rapid. The partial purification of ChAT takes one day and results in an enzyme batch for more than 4000 determinations. The endogenous Ch and ACh concentrations obtained in study I are in close agreement with those reported by other authors using microwave irradiation for killing the animals (Table II). Recently a similar approach for ACh determination was described by Massarelli et al. (1976).

3 Estimation of Brain ACh Turnover Rate *In Vivo* by Isotopic Procedures

The aim of the estimation of ACh turnover rate *in vivo* is to measure the behavior of cholinergic synapses during normal functioning as well the adaptation of the system to environmental changes. These changes can be imposed by the actions of drugs, external stimuli or perhaps by alterations in precursor availability

3 1 Basic Principles of Turnover Analysis

3 1 1 Concepts and definitions

Zilversmit and co-workers have published several articles regarding the design and analysis of isotope experiments (for review see Zilversmit 1960), and some of the concepts and definitions introduced by these authors are summarized below

Specific Activity (S) Represents the amount of radioactivity per unit of substance. It is commonly expressed as dpm per nanomole of the substance measured.

Steady State In a situation in which the rate of entry of a molecule by synthesis or transport equals the rate of exit by breakdown or transport, the concentration of the substance remains constant and a steady state is said to exist. An increase in concentration may result from an increase in the rate of formation and/or from a decrease in the loss of the substance.

Turnover This term refers to the process of renewal of a substance in a given compartment. Renewal may take place in two different ways 1) a substance may be synthesized in a given compartment, or 2) the substance may be transported to the compartment.

Turnover Rate. The rate at which a substance (X) is being renewed in a given compartment (amount per unit of time, TR_x).

Fractional Rate Constant The turnover rate is always a function of a constant (K_x) termed the fractional rate constant and denoting the state of activity and its value implicates the proportion of the compartment that is metabolized during the unit of time considered. The turnover rate then equals K [endogenous concentration of X] (TR_x=K [X]).

Turnover Time The turnover time of a substance is the time that is required for the turnover of a quantity equal to that present in the compartment. During the turnover time only 63 % of the "old" molecules have been replaced by "new" ones (insert K_x t=1 in equation 4 below).

3 1 2 Definitions of symbols and conditions

[Ch] and [ACh] represent the total amount of Ch and ACh present in a given compartment, and Ch and ACh represent the total amount of radioactive molecules in the same compartment. Thus Ch /[Ch] = S_{Ch} and ACh /[ACh] = S_{ACh}.

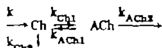
TR_{Ch} and TR_{ACh} denote the turnover rates (nmol min^{-1}) of Ch and ACh, respectively in a given compartment. The fractional rate constants for Ch and ACh are described $K_{Ch}=TR_{Ch}/[Ch]$ and $K_{ACh}=TR_{ACh}/[ACh]$ respectively. Thus the fractional rate constant is the fraction of a given compartment that is being renewed per unit of time.

It is assumed that the following conditions for Ch and ACh in brain exist

a) Free Ch in brain tissue is the immediate precursor of ACh.
b) The system is open, i.e. the isotope is continuously lost from the system by dilution with unlabelled material and by subsequent excretion.
c) A steady state exists, which means that the total rate of inflow of ACh equals the rate of outflow (release and hydrolysis, transport etc.) and this rate equals TR_{ACh}
d) The turnover rates of Ch and ACh are constant during the experimental period.
e) Random break down prevails, i.e. the brain does not distinguish between radioactive and non radioactive molecules, nor does it differentiate between newly synthesized and old molecules.
f) Ch and ACh in brain are each present in a homogeneous pool and radioactive Ch and ACh are evenly distributed in these pools.

3 1.3 Mathematical analyses

The above assumptions have been incorporated into the following model



where $K_{Ch}=k_{Ch1}+k_{Ch2}$ and $K_{ACh}=k_{ACh1}+k_{ACh2}$

The rate of entry of radioactive Ch into the Ch pool is indicated by k_p. The change of S_{ACh} will be expected to take place with time as shown in the following equation

$$\frac{dS_{ACh}}{dt} = k_{ACh}(S_{Ch} - S_{ACh}) \tag{1}$$

Thus, since K_{ACh} is constant, the change of S_{ACh} with time will depend upon the difference between S_{Ch} and S_{ACh}. The problem is to know how S_{Ch} changes with

time and thus to be able to solve equation (1). This problem can be handled in several ways

a) If it is assumed that S_{Ch} is kept constant during the experimental period, $S_{Ch}=C$ equation (1) will be

$$\frac{dS_{ACh}}{dt} = K_{ACh}(C - S_{ACh}) \tag{2}$$

which after integration gives

$$S_{ACh} \quad C\,(1 - e^{-K_{ACh} \quad t}) \tag{3}$$

Since $S_{Ch}=C$ equation (3) can be divided by S_{Ch} on both sides

$$\frac{S_{ACh}}{S_{Ch}} = 1 - e^{\ K_{ACh} \quad t} \tag{4}$$

If $S_{\ Ch}$ and S_{Ch} are measured at a given time point, K_{ACh} t can easily be calculated by means of a small desk computer

b) It may be assumed that S_{Ch} is described by a given function of time, for example when Ch is infused at a constant rate a change in S_{Ch} with time is expected to be described by the following equation.

$$\frac{dS_{Ch}}{dt} = k \ - K_{Ch} \quad S_{Ch} \tag{5}$$

After determination of k_{Ch}, $K_{\ Ch}$ can be calculated. However K_{Ch} must first be determined from multiple points during infusion (Racagni et al. 1974).

c) If the equation which describes the S_{Ch} change with time is not known, but it is possible to measure S_{Ch} and S_{ACh} at different time points after introduction of the radioactive precursor into the system, another approach can be used. The function $f(t)_{Ch}$ is assumed to be any function of time which describes the change in S_{Ch}, and this function is inserted in equation (1) to yield

$$\frac{dS_{ACh}}{dt} = K_{ACh}(f(t)_{Ch} - S_{ACh}) \tag{6}$$

Equation (6) may be integrated between two times, t_1 and t_2, as follows.

$$S_{ACht_2} - S_{ACht_1} = K_{ACh}\left[\int_{t1}^{t2} f(t)_{Ch}\,dt - \int_{1}^{t2} S_{ACh}dt\right] \tag{7}$$

The difference at the left is equal to D in Figs 4 and 6 and the quantity between the square brackets is equal to the shaded area in Figs 4 and 6 Thus K_{ACh} = D/(shaded area), which can be solved graphically or by using a computor With this method it is possible to estimate K_{ACh} and TR_{ACh} without any knowledge about induced changes in Ch kinetics.

How valid are these equations when applied to ACh turnover within the brain?

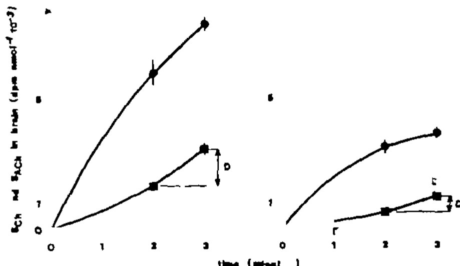


Fig. 4 S_{Ch} *and* S_{ACh} *in the striatum of rats infused with saline or unlabelled Ch for 15 min prior to an infusion of* ^3H *Ch.* ●— S_{Ch} ■—■ S_{ACh} K_{ACh} = [illegible] (from paper IV)

Unknown components are the nature and number of various "pools" of Ch (paper I) and ACh (Jenden et al. 1974 Nordberg 1977a). It is also known, at least *in vitro* that newly synthesized ACh is preferentially released (Collier 1969 Potter 1970, Molenaar and Polak 1976), which thus disagrees with condition e) under paragraph 3 1.2 above. However published data nevertheless appear to fit the model described above, at least during the first 20 min after labelling (Jenden et al. 1974 Saelens et al. 1973 Cheney et al. 1975).

3 2 Various Isotopic Approaches Currently Used to Study ACh Turnover *In Vivo*

The observation that ACh in the brain can be labelled with intravenously injected radioactive Ch (Dross and Kewitz 1966 Schuberth et al. 1969) contributed to the development of the isotopic approach to the study of brain ACh turnover *in vivo* The first application of intravenous injection of labelled Ch for this purpose was made by Dross and Kewitz (1966) and pioneer work in the development of a useful method for the estimation of ACh turnover *in vivo* was carried out by Schuberth et al. 1969 The calculations of TR_{ACh} in this method were based on the assumption that an intravenous pulse injection rapidly (within seconds) gives a constant S_{Ch} in brain (in addition to the general assumption for analysis of isotopic experiments given in paragraphs 3 1.2) and that the following relationship

exists during the first minutes after the injection. TR_{ACh}=ACh [Ch]/Ch t. Since TR_{ACh}=K_{ACh} [ACh] this is an estimation of K_{ACh} t by the following formula S_{ACh}/S_{Ch}=K $_{Ch}$ t (compare with equation (4) above), which is only valid when S_{ACh}/S_{Ch} quotients are small (for example at S_{ACh}/S_{Ch}=0.2 the calculated K_{ACh} t value is 10 per cent too low and at S_{ACh}/S_{Ch}=0.5 K $_{Ch}$ t is about 30 per cent too low as compared with the values obtained when equation (4) is solved for K_{ACh} t). What also may hamper this method is the fact that S_{Ch} in brain is not constant following an intravenous pulse injection (initial half life ~30 s, paper I) and that drug induced changes in Ch kinetics (Nordberg 1977b) may result in misleading alterations in calculated TR_{ACh}. However this method has been used successfully in several investigations in which the effect of drugs on relative changes in ACh synthesis has been studied (Schuberth et al. 1969 Schuberth al 1970, Aquilonius et al. 1973b, Nordberg and Sundwall 1975 Nordberg and Sundwall 1976, Nordberg 1977a).

Jenden and co-workers (Jenden et al. 1974) used the following variant of equation (1) for their mathematical analysis of experimental data obtained after a pulse injection of deuterium-labelled Ch dACh /dt=TR_{ACh} (S_{Ch} S_{ACh}) (equation (1) multiplied by [ACh]). TR_{ACh} was calculated from the slope of the line expressed when dACh /dt was plotted against (S_{Ch} S_{ACh}). However since a non tracer amount of deuterated Ch had to be injected in this study the steady-state of brain Ch would be perturbed (Racagni et al. 1975) and this should therefore caution against the validity of applying principles of steady-state tracer kinetics in calculating TR_{ACh}.

Racagni and co-workers (1974), who used a constant intravenous infusion of radioactive phosphorylcholine, which is rapidly hydrolyzed to radioactive choline, made the assumption that S_{Ch} changes with time were described by the following equation. dS_{Ch}/dt=k_a K_{Ch} S_{Ch}, which is inserted in equation (2) above.

Cheney et al. (1975), using an intravenous pulse injection of radioactive phosphorylcholine, applied the same basic equation as above (equation (2)) for calculating TR_{ACh}, but they also introduced the Ch kinetic in plasma as a transfer factor in order to compensate for drug-induced alteration of plasma kinetics.

3 3 Present Method (Paper IV)

In study IV a constant intravenous infusion of a tracer dose of tritiated Ch (^{3}H Ch) was given for 2 and 3 min before the animals were killed by focusing a beam of microwave on their heads. K_{ACh} and TR_{ACh} were calculated from equation (7), in which K $_{Ch}$ equals D/(shaded area) in Fig. 4 Under the approximation that the changes in S_{Ch} and S_{ACh} between 2 and 3 min are linear K_{ACh} and TR_{ACh} were calculated in a computer The theoretical error introduced by this approximation is negligible in consideration of the experimental error in each S_{Ch} and S_{ACh} value. The method makes it possible to calculate K_{ACh} even if the Ch kinetics is altered by infusion of unlabelled Ch or by the effect of drugs. However the system must

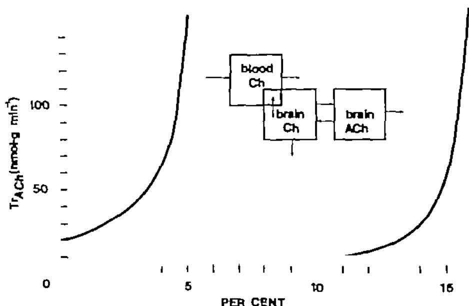


Fig. 5 "True" TR_{ACh} as a function of percentage of unequilibrated blood within cerebral blood vessels. The S_{Ch} in arterial blood were subtracted by a computer from S_{Ch} in the brain tissue homogenates prior to calculation of K_{ACh} and TR_{ACh}.

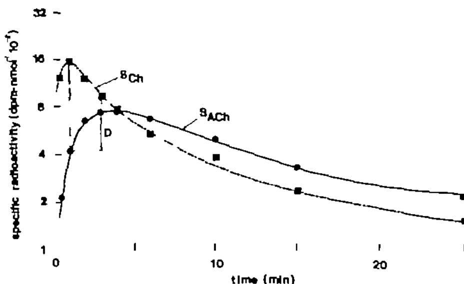


Fig. 6 Time course of S_{Ch} and S_{ACh} in brains of mice given an i.v. pulse injection of phosphoryl (Me ^{14}C) choline (data from Cheney et al. 1975).

of course still be at steady state.

When S_{Ch} in brain tissue is being measured it must be kept in mind that the brain is composed of ~94 % parenchym a and ~6 % blood, and that the S_{Ch} in arterial blood is about 10 times as high as the brain S_{Ch} during an intravenous infusion of ^{3}H-Ch(unpublished ovservatrons). Thus, if the Ch in brain blood is not completely equilibrated with the Ch in brain tissue, a brain extract can be contaminated" by a small Ch compartment of high specific activity which does not correspond to the brain Ch compartment used in ACh synthesis (Fig. 5). This will lead to too-low calculated TR_{ACh} values with all known mathematic applications for analysis of isotope experiments (Fig. 5). If the actual brain blood volume is estimated with ^{3}H-dextran (Papers I, IV), it is possible to exclude at least major changes in brain blood volume in drug experiments. However under normal conditions "contaminating blood is probably not a problem, since a product precursor relationship seems to exist between the total S_{Ch} and S_{ACh} in a brain homogenate (Jenden et al. 1974 Cheney et al. 1975 Nordberg 1977a, Fig. 6).

4 ACh Synthesis

ACh is synthesized by the transfer of an acetyl group from acetyl CoA to Ch. The reaction is catalyzed by the enzyme choline acetyltransferase (ChAT acetyl CoA choline o-acetyltranferease, E.C 2.3 1 6). This enzyme probably largely occurs in the cytosol of cholinergic neurons at physiological pH and ionic strength (Fonnum 1973).

4 1 Origin of Ch in the Brain

For optimal ACh synthesis in the perfused cervical ganglia of the rat, the Ch concentration in the perfusion medium is of great importance (Birks and MacIntosh 1961 Collier and MacIntosh 1969). It seems likely that a major portion of the Ch which acts as a substrate for ACh synthesis is Ch that is produced from ACh hydrolysis and is recaptured by the presynaptic nerve terminal (Collier and MacIntosh 1969).

Brain tissue seems to be unable to synthesize Ch *de novo* It was shown by Bremer and Greenberg (1961) that the brain has less than 1 % of the enzyme system of the liver responsible for the stepwise methylation of phosphatidylethanolamines by adenosylmethionine to form phospahtidylcholine Intracerebral injection of ^{14}C-ethanolamine in rats leads to the labelling of the ethanolamine phospholipids but not the choline lipids (Ansell and Spanner 1967). A similar procedure with C Ch lead to extensive labelling of Ch phospholipids (Ansell and Spanner 1968). *In vitro* studies (Browning and Schulman 1968) also substantiate this view as brain tissue is unable to synthesize Ch from unmethylated precursors such as serine and aminoethanol. Thus, the brain should be dependent upon an exogenous supply of Ch from plasma.

Up to now free Ch in plasma (Schuberth et al. 1969 Sparf 1973 Hanin and Schuberth 1974) and plasma lysophosphatidylcholine (Illingworth and Portman 1972) are the only established precursors of brain Ch *in vivo* (Fig. 7). However part of the free Ch in brain is probably split off enzymatically from Ch-containing compounds in the brain tissue (Dross and Kewitz 1972, Kewitz et al. 1973 Choi et al. 1975 Browning 1976), which thus might explain the negative arteriovenous difference of free Ch over the brain demonstrated in different species (Kewitz et al. 1973 Aquilonius et al. 1975 Choi et al. 1975).

There are probably two different Ch generating pools, as when a 30 min infusion of labelled Ch is stopped, the total quantity of labelled Ch leaving the brain greatly exceeds the quantity found unbound in the brain (Choi et al. 1975), indicating that a short-term infusion has labelled a source capable of generating Ch. However the Ch formed *post mortem* (Kewitz et al. 1973) is not labelled following short term infusion (Choi et al. 1975) or after acute feeding of deuterated Ch to mice (Hanin and Schuberth 1974).

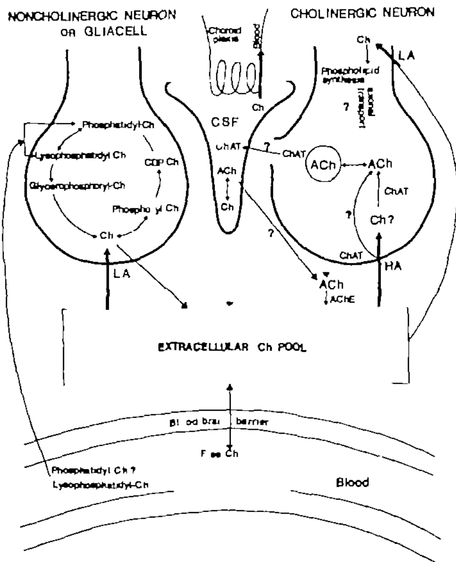


Fig. 7 Schematic diagram of the uptak compartmentation and met bolism of Ch in the brain. Abbreviations in text.

Recently Kewitz and Pleul (1976), on the basis of the observation that the S_{Ch} in brain was higher than S_{Ch} in blood after administration of labelled methionine and etahnolamine but not after administration of radioactive Ch, suggested that a *de novo* synthesis of Ch occurred in brain. However the authors have not considered the fact that lysophosphatidylcholine in plasma, which is relatively more strongly labelled following the administration of methionine and ethanolamine than after Ch injection (Kewitz and Pleul 1976), can act as a precursor for brain Ch (Illingworth and Portman 1972).

4 1 1 The fate of Ch in plasma

The half life of Ch in plasma is extremely short (Bligh 1953 paper I Freeman et al. 1975 Haubrich et al. 1975a). The clearance of the pool into which the infused Ch is distributed has been estimated to be 75 ml kg^{-1} in the rat (Freeman et al. 1975). The steady state level of plasma Ch is rapidly attained following a constant infusion of Ch and this steady state level seems to be linearly related to the infu sion rate (Gardiner and Paton 1972, Freeman et al. 1975 Gardiner and Gwee 1974 paper IV). It also appears that the endogenous plasma concentration of undeuterated Ch is not affected by a large exogenous load of deuterated Ch (Freeman et al 1975 Choi et al. 1975), which indicates that the efflux of Ch into plasma does not appear to be subject to feedback inhibition. The short half life of Ch at physiological plasma concentrations is due to a rapid uptake into tissues and subsequent metabolism to phosphorylcholine, phospholipids and betaine (Bligh 1953 Sparf 1973 Gardiner 1974 Haubrich et al. 1974 Haubrich et al. 1975a).

Since very little radioactive Ch is detected in the urine after intravenous ad ministration of labelled Ch in tracer amounts (Sparf 1973 Gardiner and Paton 1972), little regulatory contribution has been attributed to the kidney until recent ly Acara (1975) showed in chickens that the extraurinary clearance of Ch decreased, while the contribution of renal clearance to total clearance of plasma Ch increased when plasma concentrations were elevated by an infusion of Ch. At a plasma Ch concentration of 500 μM 1 the kidneys removed more than 70 % of the infused Ch. The author suggested that the renal tubule has a bidirectional transport mechanism such that net tubular reabsorption occurs at low plasma Ch levels and net tubular excretion at high plasma levels of Ch.

4 1.2 Regulation of human plasma Ch

Since the work of Bligh (1953) in which he concluded that it was unlikely that any variation in the level of plasma Ch could occur under normal and pathological conditions, little attention has been paid to the regulation of plasma Ch in man. Aquilonius and Eckernäs (1975) showed that large oral doses of Ch (3–9 g daily) resulted in a long lasting elevation of plasma Ch in patients with Hunting ton s chorea, and in view of the presence of 500–900 mg Ch in an average daily diet, a dietary influence on plasma Ch could be expected.

In study II a dietary influence on free Ch in plasma was demonstrated In 20 out of 23 fasting volunteers the plasma Ch concentration (10.6 μmol l^{-1}) increased by 1–2 μmol l (11 5 μmol l) following a meal (Table I). Hitherto few data are available on plasma Ch levels in disease or during malnutrition, and nothing is yet known about the clinical significance of any such alterations. Recently a plasma Ch concentration about twice that of normal persons was reported in azotemic subjects before hemodialysis (Remmick et al. 1976), a finding recently confirmed in our own laboratories. (Aquilonius, Eckernäs and Sahlström, to be published).

4.2 Uptake of and Compartmentation of Ch

Schuberth et al. (1969 1970) demonstrated that labelled Ch in the blood is taken up into the brain and is rapidly converted to ACh, an observation which has been verified by a number of investigators (see Freeman and Jenden (1976)). As pointed out earlier free Ch in plasma is not the only source of brain Ch Plasma lysophosphatidylcholine, for example, is able to pass the blood brain barrier In fact, and 20 % of this seems to be metabolized to free Ch in brain (Illingworth and Portman 1972). The proportions of free and derived Ch from plasma that act as precursors for free Ch in brain show species differences. In the rat derived Ch seems to contribute almost 90 % of the Ch used for ACh synthesis, while the corresponding figures in the rabbit and mouse have been calculated to be 50 % and 20 %, respectively (Schuberth and Jenden 1975).

Diamond (1971) and Sparf (1973) reported the observation of a saturable uptake of plasma Ch into mouse brain, while Freeman et al. (1975) recently suggested that a non saturable uptake of plasma Ch into rat brain existed. However in the latter investigation the plasma Ch concentration was increased to a maximum level of about 10 000 nmol ml The observed Ch concentration in brain at this plasma concentration was only about 100 nmol g Since about 6 % of the brain weight consists of blood, the observed increase in brain Ch concentration (1 % of the plasma concentration) may be a consequence of unequilibrated blood within the cerebral blood vessels.

Regardless of the original source of free Ch in brain (free Ch in plasma, Ch containing compounds in plasma and Ch liberated from Ch-containing compounds within the brain) the Ch used in the synthesis of ACh appears to be free Ch that is extracellular (Fig. 7) to the site of synthesis (see Barker 1976). This Ch pool seems to be fairly homogeneous, at least on mathematical grounds. For example in the mouse, where about 80 % of brain Ch is derived from free Ch in plasma, the S_{Ch} and S_{ACh} curves following and intra enous injection of labelled precursors (Jenden et al. 1974 Cheney et al. 1975 Nordberg 1977a) seem to fulfil the criteria for brain Ch being an immediate precursor to brain ACh in a homogeneous compartment (Fig. 6, Zilversmit 1960).

All cell membranes appear to possess a low affinity (L.A.) Ch uptake system which supplies Ch for the synthesis of phosphorylcholine (see Barker 1976), while a sodium-dependent high affinity (H.A.) system is specifically associated with the cholinergic nerve terminals and supplies Ch for the synthesis of ACh (Haga and Noda 1973 Dowall and Simon 1973 Yamamura and Snyder 1973 Suszkiw and Pilar 1976, Fig 7). It has been suggested that the H.A. Ch transport is physically coupled directly to the synthesis of ACh, and that Ch used for the synthesis of ACh thus may not have a free existence within the cholinergic nerve terminals (Barker 1976). However there is also evidence that uptake and synthesis can be kinetically coupled and that a free Ch pool might exist within the cholinergic neuron (Suszkiw and Pilar 1976). No distinction can be made between the two models with the data available today

4.2.1 Cortical and striatal *in vivo* uptake and metabolism of plasma Ch (Paper I)

In study I was observed that the quotient between brain ^{3}H-Ch and blood ^{3}H-Ch increased linearly with time following an intravenous injection of ^{3}H-Ch, both in the cortex and in the striatum (1.2 % and 6.4 % per min, respectively), thus reflecting a net transport from blood to brain (Fig. 8). The results were explained as a consequence of differences in density of cholinergic neurons between cortex and striatum. This theory is supported by Carrol and Buterbaugh (1975), who have studied the kinetics of Ch accumulation by synaptosomes prepared from different regions of guinea-pig brain. They suggested that the maximal velocity (V_{max}) of the H.A. system reflects the density of cholinergic nerve endings within brain regions. However, in view of the suggested coupling between ACh synthesis and Ch transport (see above), it would be tempting to propose that the rate of Ch uptake might reflect the rate of ACh turnover in different brain regions. In view of the turnover rate data obtained in study IV (cortex 3.6 nmol g^{-1} min^{-1}, striatum 23.8 nmol g^{-1} min^{-1}) the accumulation rates of Ch observed in study I may reflect regional differences in cholinergic activity.

As pointed out earlier it has been suggested that free Ch may not have a free existence within the cholinergic neurons (Barker 1976). If this holds true the accumulation of ^{3}H-Ch should be regarded as an "affinity" (i.e. there is a greater probability of ^{3}H-Ch derived from released ^{3}H-ACh being recaptured) for Ch rather than as a transport into the neurons. In the "free pool" model suggested by Suszkiw and Pilar (1976) the accumulation of ^{3}H-Ch could be explained by a compartmentation of Ch within the cholinergic nerve terminals. However, a combination of "affinity" and intraneuronal compartmentation is also possible (Fig. 7).

In study I, marked differences in the utilization of ^{3}H-Ch within the brain regions were also demonstrated. Cortical ^{3}H-Ch was converted into metabolites other than ACh (phosphorylcholine) at a rate of 36 % per min, while the corresponding figure in the striatum was 6 % per min. To explain these differences it was necessary to assume the existence of different "pools" of free Ch in the brain, one localized close to the H.A. uptake system and another close to the L.A. system. However, as pointed out earlier, recently published data indicate that brain Ch seems to be distributed to a fairly homogenous extracellular pool and a possible alternative explanation is thus, that in the cortex, where the value of TR_{ACh} is about six times lower than that in the striatum, there should be a six times higher probability that Ch is taken up by the L.A. system and thus mainly used in phospholipid synthesis.

4.3 Regulation of ACh Synthesis

Experiment utilizing the cholinergic system in the periphery (McIntosh 1963, Collier and McIntosh 1969, Potter 1970, Hebb 1972, Collier and Katz 1974) and in the brain (Rommelspracher and Kuhar 1974) have revealed that ACh synthesis

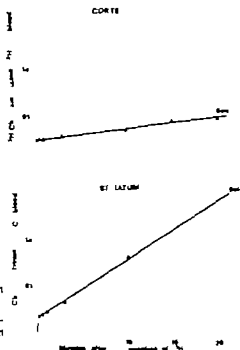


Fig. 8 Time course of the quotient between free H-Ch in blood and in brain tissue following an i. injection of tracer H-Ch. Cortex k=0.012, =0.99 striatum k=0 064, r=0.98 (from paper I).

is coupled to ACh release. Most models used to explain this coupling have involved ChAT The models include feedback inhibition of ChAT by ACh (Kaita and Goldberg 1969 Morris et al. 1971) and mass action regulation of ChAT (Glover and Potter 1971). However there has been no direct evidence that ChAT is regulated *in vivo*

Recent observations indicate that the availability of Ch is rate limiting in ACh synthesis (Eisenstadt and Schwartz 1975 Simon et al. 1976). In view of the presumed coupling between H.A. Ch uptake and ACh synthesis described above, an attractive hypothesis would be that the H.A transport system has a regulatory function. It was thus of great interest when Simon et al. (1976) demonstrated that alterations in the activity of cholinergic pathways *in vivo* caused by lesions or drugs resulted a change in V_{max} for the H.A. transport of Ch when tested *in vitro* For example treatment with drugs that increase neuronal activity e.g. pentylenetetrazol or scopolamine, cause an increase in V_{max} of H.A. transport into brain synaptosomes without any change in K_m. Conversely decreased neuronal activity after pentobarbital, or lesion causes a decrease in V_{max}.

Jenden et al. (1976) reported a correlation between the rate of H.A uptake of Ch and the ACh content of synaptosomes, and suggested that the synaptosomal ACh concentration might regulate H.A. Ch uptake. However Kuhar et al. (1975) found that ACh concentrations in the interpeduncular nucleus did not change after acute lesions or pentobarbital administration, but the Ch uptake in that region was nevertheless reduced. Thus, it remains to be established whether

other regulatory mechanisms exist.

Some reported results do not seem to be explainable on the basis of the H.A. system alone, and in these cases the L.A. system may play a role. For example, it has recently been demonstrated that parenteral administration of Ch can result in increased plasma and brain Ch concentrations and in brain ACh concentration (Haubrich et al. 1974 1975 Cohen and Wurtman 1975). Since the sodium dependent H.A Ch uptake (K_m=0.75 μM Simon et al. 1976) would already be saturated, it seems that the L.A. Ch uptake could subserve ACh synthesis, since the L.A. system has a K_m value of about 50 μM (Yamamura and Snyder 1973 Barker and Mittag 1975 Carroll and Goldberg 1975) and would not be saturated at normal Ch levels. Carroll and Goldberg (1975) suggest, on the basis of experiments *in vitro* that when the nerve terminals are depolarized, Ch can be supplied by the L.A. transport system for the synthesis of ACh. This is in contrast to the finding of Simon et al. (1976) however that altered neuronal activity only affected the V_{max} of the H.A. transport system for Ch. Furthermore, Suszkiw and Pilar (1976), who studied synaptosomes from the ciliary cells in the iris, suggested *that cholinergic nerve terminals seem to be deficient in the L.A transport system*, while the cell bodies of the same neurons are deficient in the H.A. system. It remains to be established however whether this is a general property of all cholinergic neurons.

4 3 1 Effect of elevated plasma Ch concentration on *in vivo* turnover rate of brain ACh (Paper IV)

The aim of the present paper was to evaluate the proposal recently put forward by several authors (Cohen and Wurtman 1975 Haubrich et al. 1975b, Wurtman and Fernstrom 1976) that increased concentrations of plasma Ch stimulate the synthesis of brain ACh. At steady state an increased transmitter synthesis must be balanced by an increased transmitter outflow and thus result in an increased turnover rate of the transmitter (Zilversmit 1960).

Rats were infused intravenously with saline (0.25 ml min kg^{-1}) or unlabelled Ch (15 μmol min kg^{-1}) for 15 min and an infusion of ^{3}H-Ch was then started (Fig. 9). The two infusions were then continued simultaneously for a further two or three minutes. During the infusions the animals were held in place in the holder within a microwave equipment (drawn very schematically in Fig. 10). The rats were killed by focusing a beam of microwave on their heads 2 and 3 min after the ^{3}H-Ch infusion was started. TR_{ACh} was calculated as described in paragraph 3.3 above.

The elevated steady state concentrations of plasma Ch (~140 μmol l^{-1}) were of the same magnitude as the peak values shown to be paralleled by increased brain ACh concentrations in the studies by Haubrich et al. (1975) with use of intraperitoneal administration of Ch.

At the elevated plasma Ch level the Ch concentration was significantly increased both in the cortex and in the striatum, while the ACh concentrations were

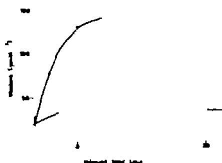


Fig. 9 Time course of plasma Ch in rats infused with unlabelled Ch. ▼–▼ 15 µmol kg⁻¹ min⁻¹ ▽–▽ 2.5 µmol kg⁻¹ min⁻¹ (from paper IV).

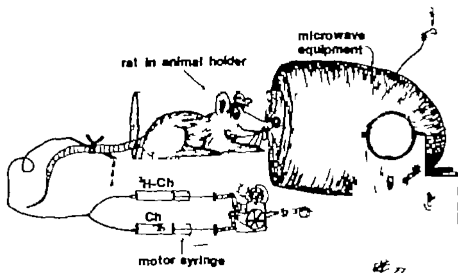


Fig. 10 A very schematic figure of the procedure used in paper IV

Table III *Cholinergic parameters in brain regions of the rat at different steady-state concentrations of plasma Ch*

Brain part and steady-state concentration of plasma Ch	Ch (nmol g^{-1}) m ± S.E.	ACh (nmol g^{-1}) m ± S.E.	Fractional rate constant K_{ACh} (min^{-1})	Turnover rate TR_{ACh} (nmol g^{-1} min^{-1})
Cortex (plasma Ch 17 µmol l^{-1} n=18)	43 ± 2.4	16 ± 1.6	0.22	3.6
Cortex (plasma Ch 140 µmol l^{-1} n=15)	59 ± 3.7**	14 ± 1.7	0.26	3.6
Striatum (plasma Ch 17 µmol l^{-1} n=23)	33 ± 1.22	76 ± 2.9	0.31	23.8
Striatum (plasma Ch 140 µmol l^{-1} n=22)	53 ± 2.0**	84 ± 3.0	0.26	21.4

* $p < 0.02$
** $p < 0.001$ (Student's t-test)

increased slightly in the striatum only (Table III). However no conclusive changes in the regional TR_{ACh} were detected (Table III). These findings thus contradict the suggestion that increased plasma levels of Ch are parallelled by enhanced neuronal synthesis of ACh in the brain. These results are not unexpected, since it would be unlikely that neuronal functional activity should normally be regulated by its precursor availability

The slight and significant increase in striatal ACh concentration and the minute reduction of striatal TR_{ACh} might indicate a direct agonistic effect of Ch itself in some receptor-close compartment. However the present findings do not necessarily rule out a possibility of restoring cholinergic function with Ch under pathological conditions (Paper VI).

5 Cholinergic Mechanisms in the CNS

5.1 Methods for Localization of Cholinergic Neurons

Direct visualization of ACh as achieved by the histochemical fluoroescence of catecholamines seems to be impossible in view of the simple chemical structure of ACh. However a variety of methods for identifying and localizing cholinergic tracts have been developed.

5.1.1 Biochemical "markers"

Biochemical markers" include ACh levels, ChAT and AChE activity H.A. Ch uptake and the presence of cholinergic receptors (see Kuhar 1976). ACh concentration can, in animal experiments, reflect the cholinergic representation at the gross anatomical level if special precautions for stabilization of ACh have been undertaken (see above). However in human studies ACh concentrations probably cannot be used as a cholinergic marker because of the post-mortal changes.

The activity of the cholinergic enzymes ChAT and AChE can easily be measured by sensitive methods (Fonnum 1973). There is little doubt, however that ChAT is a far more relevant marker for cholinergic structures than AChE. In some brain regions, for example, there is no correlation between AChE and ACh concentration, while the ChAT activity is well correlated to ACh concentration in different regions (Koelle 1969 Nordberg and Sundwall 1975).

Sorimachi and Kataoka (1974) and Simon and Kuhar suggested that H.A. uptake of Ch as measured in synaptosomes prepared from different brain regions could be a specific marker for cholinergic neurons, but in view of the recent suggestion that the H.A. transport system is adapted to functional changes in the cholinergic neurons (Simon et al. 1976), regional differences in H.A. uptake of Ch might possibly reflect differentes in the functional activity of cholinergic neurons more than the relative density of cholinergic neurons.

Another useful biochemical marker for cholinergic representation is the presence of what appear to be the cholinergic receptors. This is a rather new area of research and will perhaps give additional knowledge about cholinergic anatomy in the future (see Kuhar 1976).

5.1.2 Histochemical methods

There are several workable methods for localization of AChE at light and electron microscopic levels (see Kuhar 1976). However as pointed out earlier AChE does not necessarily represent the cholinergic innervation. Apart from the localization of AChE in the endoplasmatic reticulum, in axons and in synaptic regions, it has

also been suggested that AChE is present in glial cells (Hemminiki et al. 1973).

The presumably unique presence of ChAT in cholinergic neurons thus makes it more useful for histochemical localization. The first method described for detection of ChAT was based on the precipitation of CoA, which is produced in the formation of ACh (and also in other biochemical reactions) as a mercaptide (Burt 1970 Kasa et al. 1970). However Burt and Silver (1973) later reinvestigated the specificity and suggested that this histochemical method was not completely reliable.

Another approach recently developed is immunohistochemical methods (Eng et al. 1974 McGeer et al. 1974). However the specificity is not very well established at present (Rossier 1975). In spite of the many problems involved and the need for extensive controls, several laboratories are intensively engaged in trials with these methods (Malthe-Sørenssen et al. 1973 McGeer et al. 1975 Singh et al. 1975 Hattori et al. 1976 Rossier 1976), and within the next few years such techniques will probably be more prominent in cholinergic research.

5 2 Distribution of ChAT in CNS

The radioenzymatic procedures for ChAT determinations permit assay in μg amounts of brain tissue (Fonnum 1973), and since ChAT has been shown to be remarkably stable post mortem (Paper V) it can be used in studies on human brain.

The first study on ChAT activity in human brains, though relatively limited, was made by Hebb and Silver (1956), who used bioassay to determine the enzyme activity They observed that the activity of ChAT showed marked regional differences, with very high values in the striatal system. Similar relative distribution patterns have also been found in lower animals (Hebb and Silver 1956). The first more detailed study in primates (Rhesus monkey) was made by Fahn and Côté (1968) and in human brain by McGeer and McGeer (1971) and Aquilonius et al. (1973). However at this stage all tissue samples consisted of gross anatomical regions removed by free-hand dissection.

5 2.1 Regional distribution of ChAT in human brain (Paper V)

Until recently it was necessary to dissect fresh brain to obtain tissue samples. This method is very inaccurate, the main drawback being that the sample from the area of interest is usually very poorly defined. Possible subnuclear differences in analysed substances in an apparently homogeneous structure cannot be detected.

In paper V a method for obtaining well-defined samples from brain tissue was developed and was applied to ChAT analysis in subregions of human brain. From a frozen brain oriented so that 200 μ cryo sections are taken (LKB 2250 PMW Cryo-Microtome, LKB Produkter AB, Sweden) perpendicular to the intercommissural plane (anterior and posterior commussures)and to the sagittal plane. Tissue samples (~0.1 mm^3) are then punched out, using a small glass capillary



Fig. 11 The distribution of punchings in a typical section of human brain, 5 mm behind the anterior commissure (from paper V).

(Fig. 11), and the ChAT activity is measured by a radiochemical procedure. The enzyme activity is expressed in relation to unit of volume of brain tissue, which is probably better than wet weight, since the area punched out (and thus original volume of tissue) will not change even if moisture is lost (or added).

Several anatomical regions could be subdivided according to differences in ChAT activity (Fig. 12, Table IV). Especially in the caudate nucleus different populations could be traced sterically through the sections. Little is known as yet about possible physiological correlates to these subnuclear regions, but it may be of interest to note that choreiform hyperkinesia in the cat is induced after local application of dopamine only in the rostromedial part of the caudate nucleus (Cools 1972).

The extreme range (10–140 nmol ACh/min-cm^3) of ChAT activity found in the amygdala indicates that some subnucleus might be rich in cholinergic neurons. The highest values seemed to be in the central part of the nucleus. However no statistically significant regions could be distinguished owing to the relatively fewness and scattering of the punchings. Most of our findings in study V concerning intranuclear distribution of ChAT in human brain have recently been confirmed by McGeer (1976).

5 3 Some Well Established Central Cholinergic Tracts

ChAT is synthesized in the cell body of the cholinergic neuron and is transported by axonal flow to the terminal region. Accordingly a lesion of a cholinergic


C
+5
C
5
C
10
C
-20
Ve l
Cp l
0
CAT activity (nmoles ACh formed/minute per tissue)
1
236 240

Table IV *ChAT activity in different regions of human brain*

Structure	Population area	ChAT activity (nmole ACh/min cm^3) ±SE ( )	No
Caudate nucleus	Cd1	108.7 ± 8.3	13
	Cd2	194.1 ± 4.0	98
	Cd3	151.3 ± 6.5	43
	Cd4	91.8 ± 4.7	27
Putamen	Pt1	237.5 ± 3.9	147
	Pt2	319.3 ± 7.6	34
	Pt3	180.4 ± 16.5	9
Globus pallidu lateralis	Pl 1	12,4 ± 1.0	15
	Pl 2	8.7 ± 0.3	41
	Pl 3	15.1 ± 1.8	31
Globus pallidu medialis	P.m	4.5 ± 0.2	31
Nucleus ansa lentiformis	A.l	35.4 ± 7.1	14
Claustrum+5	Cl	4.9 ± 0.4	9
Claustrum−5 −10 −20	Cl	10.4 ± 0.5	35
Substantia nigra pars compacta	NI.c	5.9 ± 0.5	11
Substantia nigra pars reticulata	NI.r	3.2 ± 0.1	15
Subthalamic nucleus	Sth	12.2 ± 0.9	15
Nucleus ruber	Ru	5.0 ± 0.1	2
Thalamus medial part	Th.m	10.5 ± 0.6	33
Thalamus lateral part	Th.l	6.4 ± 0.3	73
Substantia reticularis thalami	Rt	4.9 ± 0.3	15
Amygdala	Am	25.6 ± 2.7	61
Gyrus cinguli	G.c	4.5 ± 0.1	5
Gyrus olfactorius	G.ol	10.0 ± 0.8	13
Insula	Cx.In	8.6 ± 0.6	6
Hippocampu	Hipp	13.6 ± 1.5	17
White substance	W	1.9 ± 0.1	14

Fig. 12 Normal distribution of choline acetyltransferase (ChAT) in different regions of human brain (5 mm rostral and 5, 10 and 20 mm behind the anterior commissure). Abbreviations: A.l—nucleus ansa lenticularis, Am—amygdala, Cd—caudate nucleus, C.c—corpus callosum, Cl—claustrum, Cm.a—anterior commissure, Cp.l—internal capsule, Cx.In—insula, Fx—fornix, G.c—gyrus cinguli, G.ol—gyrus olfactorius, Hipp—hippocampus, NI.c—substantia nigra pars compacta, NI.r—substantia nigra pars reticulata, Pl—lateral pallidum, Pm—medial pallidum, Pt—putamen, Rt—substantia reticularis thalami, Ru—nucleus ruber, Sth—subthalamic nucleus, Th.l—thalamus, lateral part, Th.m—thalamus, medial part, w—white substance, White areas—not analysed. (From paper V).

neuron is accompanied by accumulation of ChAT proximal to the lesion and depletion of the enzyme in the target area of the neuron.

It is well known that a septal-hippocampal pathway exists (see Kuhar 1976), and since some of these neurons stain for AChE (Lewis and Shute 1967) a cholinergic septal-hippocampal parthway might be present. Consequently lesions in the fimbria result in a large decrease of AChE and ChAT in the hippocampus on the lesioned side (Lewis et al. 1967 McGeer et al. 1969) It has been possible to localize ChAT activity to certain cell layers of the dentate area of the hippocampus (Fonnum 1970) Moreover Kuhar et al. (1973) found that lesions in the medial septal area resulted in a sharp reduction in the H.A. uptake of Ch into crude synaptosomal fractions prepared from the hippocampus, and in a concomitant decrease in activity of ChAT

Following lesions of the major known afferent parthway to the caudate nucleus in rats no decrease in AChE, ChAT (McGeer et al. 1971a) or ACh (Butcher and Butcher 1974) could be detected in the striatum. Furthermore, when the striatum is destroyed no alteration of ChAT is found in other brain regions (McGeer et al. 1969). These findings suggest that striatal cholinergic neurons mostly are interneurons.

Lesions of the crossed and uncrossed olivocochlear bundle in the cat are accompanied by a 90 % reduction of ChAT activity in the membranous cochlea (Jasser and Guth 1973), thus supporting the existence of a cholinergic efferent tract from the superior olivary complex to the cochlea hair cells.

It is now fairly well established that some of the neurons in the habenulo-interpeduncular tract are also cholinergic (see Kuhar 1976).

5.4 Interaction of Cholinergic Neurons with Other Neuronal Systems in Extrapyramidal Nuclei

The neostriatum has one of the highest densities of cholinergic neurons found in the CNS and, as discussed earlier there is considerable evidence to suggest that these are interneurons (McGeer et al. 1971a, Butcher and Butcher 1974 McGeer et al. 1975 Hatton et al. 1976). About 95 % of all neurons in the neostriatum seem to be Golgi type II interneurons (Kemp and Powell 1971). The neostriatum also contains dopaminergic terminals originating in the zona compacta of the substantia nigra (Andén et al. 1964 Dalström and Fuxe 1964 Ungerstedt 1971). In the rat it has been estimated that there are about 3 500 dopaminergic neurons in the substantia nigra which produce about 1 6 x 10 nerve terminals in the neostriatum (Andén et al. 1966).

It has long been known that iontophoretic application of ACh enhances the firing rate of cells in the caudate, whereas dopamine depresses the majority of these cells (Bloom et al. 1965). In neuropharmacological experiments dopamine agonists decrease ACh release and turnover in the striatum while dopamine

Table V *Effect of various treatment on cholinergic parameters in the striatum*

Treatment	ACh levels	ACh release	ACh turnover
Dopamine agonists	↑(1 2 9)[a] ↕(4)	↓(6)	↓(4)
Dopamine receptor blockers	↓(1 8), ↕(4)	↑(6)	↑(4)
Muscarinic recept stimulators (Ox tremorin)	↑(4 5)	–	↓(4 5)
Muscarinic receptor blockers	↓(4)	↑(11)	↕(4)
GABA receptor blockers (Picrotoxin)	↑(10)	–	
Lesion f the medial forebrain bundle	↓(7)	–	
Electric stimulation f the nigro-neostriatal path ay	–	↓(6)	–

↑ increase

↓ decrease

↕ no change

[a] The number in the parentheses indicate reference source of d ta

(1) Paper I
(2) Consolo t al (1974)
(3) Hambrech et al. (1975c)
(4) Racagni t al. (1976)
(5) Nordberg and Sundwall (1977)
(6) Bartholini t l (1976)
(7) Rommelspacher and Kuhar (1975)
(8) Sethy and Van Woert (1974)
(9) Schmidt (1976)
(10) Ladinsky t al. (1976)

receptor blocking agents increase ACh release and turnover in this region (Table V). Thus, it seems likely that a dopaminergic input from the substantia nigra has a tonic inhibitory influence on some of the cholinergic interneurons in the neostriatum (see Roth and Bunney 1976).

Data have also been presented that support a theory of a cholinergic system in the substantia nigra (see Roth and Bunney 1976). The origin of the cholinergic system in the substantia nigra may be intrinsic or extrinsic, but in the latter case all that can be said at this time is that is does not arise from the striatum (Hatton et al. 1973 Kataoka et al. 1974 Fonnum et al. 1974).

There is now substantial biochemical evidence of a γ-aminobutyric acid (GABA)-ergic input to the substantia nigra originating in the striatum. A lesion in the strionigral pathway results in a marked decrease in both GABA and glutamic acid decarboxylase in the substantia nigra (Kim et al. 1971 McGeer et al. 1971b, Hatton et al. 1973 Fonnum et al. 1974). Recently Ladinsky et al (1976) observed that the ACh concentration in rat striatum increased after ad ministration of the GABA-ergic blocking agent picrotoxin. This supports a theory of a GABA ergic (inhibitory)—dopaminergic (inhibitory)—cholinergic link ter

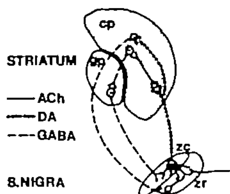


Fig. 13 Possible interactions between cholinergic, dopaminergic, and GABA ergic neurons linking the substantia nigra and basal ganglia. cp—caudate nucleus and putamen, gp—globus pallidus, zc—zona compacta of substantia nigra, zr—zona reticulata of substantia nigra.

minating in the striatum. In Fig. 13 a possible model for the interactions between cholinergic, dopaminergic and GABA-ergic neurons in the substantia nigra and basal ganglia is depicted.

6 Huntington's Chorea

6.1 Introduction

Huntington's chorea (H.C.) is a hereditary disorder of unknown etiology characterized by the onset of progressive choreiform movements and dementia usually between the third and fifth decades of life.

The first detailed description and the first account of the hereditary nature of *the disease were given on 15th February 1872 by the 22 year-old George Huntington* in a lecture before the Meigs and Mason Academy Middleport, Ohio.

The disease is inherited in an autosomal dominant fashion with complete penetrance (see Barbeau et al. 1974) and involves characteristic degeneration of small (Golgi type II) neurons in the neostriatum (Bruyn 1968).

6.2 Rôle of Different Transmitter Systems

6.2.1 Cholinergic system

Several clinical observations compatible with reduced activity of cholinergic neurons in H.C. have been made. It has been demonstrated that central cholinergic stimulation by physostigmine reduce choreatic movements, while blockade of central cholinergic receptors aggravates the symptoms (Aquilonius and Sjöström 1971 Klawans and Rubovits 1971 1972, Davis et al. 1976). Aquilonius et al. (1972) found decreased concentrations of Ch in the lumbar CSF of H.C. patients, which might be reflective of cholinergic dysfunction. Furthermore, there are marked reductions of the cholinergic marker ChAT in the neostriatum of H.C. patients with this disease (McGeer et al. 1973 Aquilonius et al. 1974 Bird and Iversen 1974 Stahl and Swanson 1974 Paper V), especially in the rostromedial part of the caudate nucleus (Fig 14 Paper V). The morphological basis of the cholinergic hypofunction in H C. thus seems to be a degeneration of striatal cholinergic interneurons. However loss of muscarinic receptors in the caudate and putamen has also been reported (Hiley and Bird 1974 Wastek et al. 1976). At present, it is not known whether this is primary or secondary to the *degeneration of cholinergic neurons.*

6.2.2 Catecholaminergic system

There is some contoversy regarding involvement of a dopaminergic degeneration in H.C. Unchanged activity of tyrosine hydroxylase, a marker for dopaminergic and noradrenaline terminals, has been found in the striata of H.C. brains (McGeer et al. 1973). Normal levels of dopamine (DA) and homovanillic acid have also been ovserved by several investigators (Ehringer and Hornykiewicz 1960, Hor

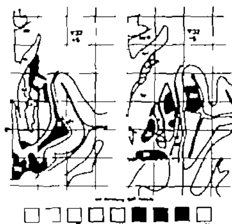


Fig. 14 Brain ChAT (per cent of control values) in a patient with Huntington's chorea. Abbreviations as in Fig. 12. White areas: not analysed (from paper V).

nykiewicz 1966, Bird and Iversen 1974 Mattson 1974), while decreased concentrations of dopamine limited to the caudate were found in one study (Bernheimer and Hornykiewicz 1973).

In an investigation by Lal et al. (1973) the increase in homovanillic acid in CSF following probenecid administration was found to be similar in controls and H C. patients and may be taken as an indication of a preserved dopaminergic mechanism in H.C. Thus, most data available at present indicate that this mechanism are relatively intact in H.C.

6 2.3 GABA-ergic system

As discussed earlier there is evidence of a DA ACh-GABA linkage between the substantia nigra and the striatum. It is thus interesting to note that deficits in the GABA concentrations in the neostriatum and substantia nigra (Perry et al. 1973) and markedly decreased levels of GABA in CSF (Glaeser et al. 1975) have been found in H.C. A significant reduction of glutamic acid decarboxylase in the striatum of H.C patients has also been reported (Bird et al. 1973), McGeer et al 1973 Bird and Iversen 1974 Stahl and Swanson 1974 Urguhart et al. 1975). However glutamic acid decarboxylase deficits are not specific to H.C. and are also seen Parkinson's disease (Lloyd and Hornykiewicz 1973 Laaksonen et al. 1974) and in ageing (Bowen et al. 1974 Urguhart et al. 1975). Further the decrease in glutamic acid decarboxylase in H.C seems to be uniform (McGeer et al. 1973b) in striatum and substantia nigra, while ChAT reductions are patchy (McGeer et al. 1973, Paper V).

6.2 4 Conclusion

It may thus be suggested that in H.C the DA ACh GABA-ergic loop in the basal ganglia might be disturbed owing to degeneration of cholinergic interneurons in the striatum and possibly a parallel or secondary degeneration of GABA-ergic neurons from the striatum to the substantia nigra.

To understand the effect of such degenerative changes it may be relevant to introduce the term "biological buffer capacity" (Gerlach et al. 1974) to express the ability of a neuronal system to maintain normal function under different environmental conditions. Thus, in H C it can be assumed that the "buffer capacity" of the basal ganglia is decreased, at least in some parts to a level where choreatic movements appear spontaneously. In asymptomatic relatives with the inherited gene for H.C., provocation with L DOPA can induce hyerkinesia in some cases (Klawans et al. 1972). This could thus be explained by a less pronounced decrease in "buffer capacity" and concequently the hyperkinesia only being manifested when the system is disturbed.

However much more basic studies will be required to delineate the relative importance of different transmitter systems during normal and abnormal function of the basal ganglia.

6 3 Different Therapeutic Approaches

No curative treatment for the progressive dementia and motor disturbances is known at present and all current therapy is symptomatic.

6.3 1 Drugs affecting the cholinergic system

6.3.1.1 Cholinesterase inhibitors Several investigators have shown that physostigmine, a centrally active anticholinesterase, reduces the choreatic movements in H.C. patients (Aquilonius and Sjöström 1971 Klawans and Rubovits 1971 1972 Davis et al. 1976). However not all patients seem to be improved by physostigmine. In a study by Tarsy et al. (1974) the chorea was reduced in only two out of twelve patients. Davis et al. (1976) found that one out of four patients was unaffected by physostigmine, and Fahn et al. (1973) saw no improvement in any of four patients treated with physostigmine.

6.3.1.2 Choline It has been demonstrated that parenteral administration of Ch to animals may result in increases in plasma and brain Ch concentrations and in brain concentrations of ACh (Haubrich et al. 1974 1975 Cohen and Wurtman 1975 Paper IV see above). This finding was of course of great interest as it suggested that Ch could be used as a "cholinergic L DOPA" in the treatment of postulated hypocholinergic disorders such as H C and tardive dyskinesia.

In 1975 it was shown by Aquilonius and Eckernäs that oral administration of Ch to patients resulted in a long-lasting elevation of the plasma Ch concentration, and in the same year Davis et al. (1975) reported that 16 g of Ch per day had beneficial effect in a case of tardive dyskinesia.

In study VI the effect of high oral doses of Ch (15 g/day) on the hyperkinesia in five patients with H.C was examined. In spite of the dose-dependent increase in plasma Ch (Fig. 15), little or no clear therapeutic effect was noted following the

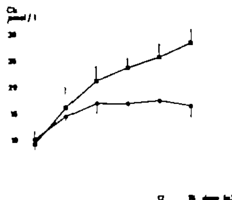


Fig. 15 Plasma Ch in five patients on oral doses of Ch. Plasma samples were taken on the third day following a change in Ch dose. ●—● 16 after the last daily dose; ■—■ 1 h after the second daily dose. Vertical bars indicate S.E.M (from paper VI).

Ch treatment (Fig. 16). Two patients (AB and BZ) showed a tendency to a dose dependent reduction in their hyperkinetic movements, but only one type of hyperkinesia (no 6 in Fig. 16) in one patient (AB) returned to the premedication level following withdrawal of Ch, while the other hyperkinesias (nos. 2, 4 and 5 Fig. 16) were reduced even more after a two-month withdrawal period. One type of chorea in this patient (no. 5) even disappeared during the withdrawal period and a new type of hyperkinesia appeared instead. Such a spontaneous change in the hyperkinetic pattern might of course give rise to misleading interpretation of results when long-lasting drug effects in H C are being studied.

The failure to affect the choreatic movements by Ch treatment can be explained in several ways. Firstly as shown in Paper IV there is no indication of an increased turnover rate of ACh in brains of normal animals when plasma concentrations of Ch are increased. Thus, residual normal neurons in the brain of a patients with H C probably cannot increase the ACh release in order to compensate for degenerated neurons. However if the normal availability of Ch at the site of the synthetic reaction is reduced, for example, due to a defect in the H.A. system at early stages of degeneration, treatment with Ch might theoretically restore ACh synthesis. Secondly as shown in study V the degeneration of cholinergic neurons in the stratum is not uniform, and one part with complete degeneration might correspond to one type of refractory hyperkinesia. Muscarinic receptor sites might also be reduced in a patchy manner (Hiley and Bird 1974 Wastek et al. 1976). Finally the complexly disturbed interaction between different possible neuronal systems (DA ACh-GABA) cannot be restored merely by alteration of cholinergic function.

When preparing this survey a similar study was published (Davis et al. 1976), in which Ch in doses of up to 20 g daily was administered to four H C. patients. Two patients showed a dose-dependent decrease in chorea, but in view of what appears to be spontaneous fluctuation in hyperkinesia, the result must be interpreted with caution. Nevertheless, it is felt that the present data concerning the clinical effects of Ch treatment in H.C justify further studies. Since part of Ch

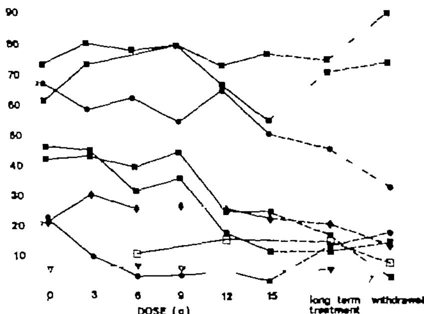


Fig. 16 Frequences of involuntary movements (movements/min) in five patients suffering from H.C. treated with oral doses of Ch.
■ BZ, ● AB, ♦ MB, □ MN ▽ PO
Types of hyperkinesia. 1 finger movements, 2. flexion of toes, 3 elevation of right leg, 4 head movements, 5. oral hyperkinesia, 6. abduction of the legs, 7 elevation of the eye brows, 8. maximal time (seconds) for extension of the tongue, 9 widening of the eyes (from paper VI).

seems to be metabolized to trimethylamine in the gut by bacterial action and in view of the possible rôle of trimethylamine in uremic toxicity (Simenhoff 1975), studies concerning possible toxicological effects of long-term treatment with high doses of Ch also seem of special importance.

6.3 1.3 2 Dimethylaminoethanol (deanol) There is much controversy concerning deanol as a "cholinergic drug Despite the evidence that Ch is formed slowly if at all, by methylation of the Ch analogues ethanolamine and dimethylaminoethanol (see above) in the brain, several investigators have described deanol as a precursor of brain Ch (Pfeiffer 1959 Walker et al. 1973 Miller et al. 1974, Miller 1974 Casey and Denny 1975, Haubrich et al. 1975b, Widroe and Heisler 1976). Some authors have reported that deanol increases brain ACh concentrations in animals (Pfeiffer 1959 Danysz et al 1967 Goldberg and Silberyeld 1974 Haubrich et al. 1975b), while others have failed to substantiate a conversion of deanol to Ch and ACh (Pepeu et al. 1960 Zahiser and Hanin 1976).

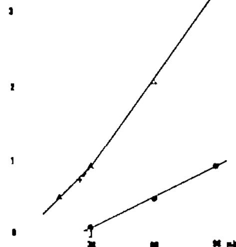


Fig. 17 Time course of ACh formation upon incubating on of human CSF ●— untreated CSF ▽—▽ boiled CSF (from paper VII)

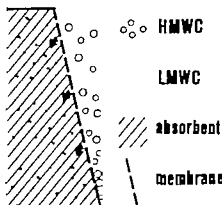


Fig. 18 The use of Minicon B15 (Amicon B.V Holland) for obtaining a concentration of high molecular weight compounds (HMWC), while low molecular weight compounds (LMWC) are diluted at the same time (used in paper VII).

Despite the controversy regarding the action of deanol there are several reports of the therapeutic efficiency of this drug in tardive dyskinesia (Miller et al. 1974 Casey and Denny 1975 Curran et al. 1975), L DOPA induced hyperkinesia (Miller 1974) and Huntington s chorea (Walker et al. 1973). Other investigators, however have failed to show any effect of deanol on tardive dyskinesia (Escobar and Kemp 1975) and in L DOPA induced dyskinesia (Klawans et al. 1975).

6 3.2 Drugs affecting the monoaminergic system

Drugs lowering (reserpine, tetrabenzines), displacing (α-methyl-dopa) or blocking (phenothiazines, butyrophenones) the action of catecholamines have all been reported to be of some use, albeit limited, in H C (see Barbeau 1975). On the other hand, L DOPA has generally been shown to aggravate chorea, except in the small number of patients who have the rigid akinetic form (juvenile form) of H C where L DOPA may improve the neurological status (see van Woert 1976).

In animal experiments it has been shown that a dopaminergic blockade increases the turnover of ACh in the striatum (Table V Trabucchi et al. 1973 Cheney et al. 1974) and this could perhaps be the mechanism underlying the improvement of hyperkinesia.



Fig. 19 Time course of ACh formation upon incubating of selectively concentrated human CSF. The high molecular weight (HMWC) fraction is concentrated 10 times while the low molecular weight (LMWC) fraction is diluted 10 times. —• unboiled CSF ▽—▽ boiled CSF (from paper VII).

6.3.3 Drugs affecting the GABA-ergic system

Several investigations have failed to show any clear-cut effect on H.C. with different GABA-ergic stimulating drugs (see Barbeau 1975).

6.4 Early Detection

One of the aims of biochemical research in H.C. is the development of a test to permit detection of the disease long before the appearance of symptoms and, if possible, before the child-bearing age. Unfortunately such a goal has not yet been achieved, but several approaches have been tried, for example the L-DOPA load test (Klawans et al. 1972), "physiological test battery" and prolactin secretion following L-DOPA (Barbeau 1975).

Since degeneration of cholinergic neurons in the CNS theoretically would give rise to altered levels of ChAT in CSF, assay of the enzyme activity in lumbar CSF might be a possible way of achieving early detection. In study VII a procedure was developed enabling the analysis of the small quantities of ChAT appearing in "normal" CSF. It was shown that a non-enzymatically catalyzed synthesis of ACh, which was increased when CSF was boiled, appeared in untreated CSF during analysis (Fig. 17). This could be overcome, however, by a specific concentration of high molecular weight compounds in the CSF by use of a membrane with selective permeability (Figs. 18, 19). The method is now being applied in an investigation of CSF from H.C. patients and relatives of such patients.

7 General Summary

Simple and rapid methods for the determination of free choline (Ch) in plasma and of Ch and acetylcholine (ACh) concentrations in brain tissue have been developed.

Basic principles for turnover analysis are discussed, and these principles were applied in estimation of the turnover rate of ACh in rat brain parts following i.v infusion of ^{3}H-Ch is described.

The origin and compartmentation of Ch in the brain and the regulation of ACh synthesis are treated. It is concluded that it seems unlikely that increased concen trations of plasma Ch are paralleled by an increased turnover rate of ACh under physiological conditions.

Different available methods for localization of cholinergic structures within the CNS are discussed. A stereotaxic method for tissue sampling in human brain was applied in a study of regional distribution of the cholinergic marker choline acetyltransferase (ChAT) in human brains. It was found that several nuclei of the basal ganglia could be subdivided according to the density of the cholinergic marker

Possible pathophysiological mechanisms involved in Huntington's Chorea (H C) are discussed. Extreme reductions of ChAT were found in basal ganglia of brains of H.C patients and it is concluded that a cholinergic hypofunction in the neostriatum due to degeneration of small cholinergic interneurons seems to be of patophysiological importance.

Patients with H.C were treated with high oral doses of Ch (15 g daily), but no clear-cut beneficial effect was noted. Some hyperkinesias showed an apparent dose-dependent reduction in frequency However this might have been an artefact due to a spontaneous variation in the hyperkinetic pattern. Nevertheless, it is felt that the present data justify further studies.

Early detection of H C before child-bearing age is an important aim. Since degeneration of cholinergic structures within the CNS theoretically should result in increased levels of ChAT in lumbar CSF a method for the determination of the enzyme activity in human CSF was developed.

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Local Sympathetic Reflex Mechanism in Regulation of Blood Flow in Human Subcutaneous Adipose Tissue

BY
OLE HENRIKSEN

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From the Department of Nuclear Medicine Rigshospitalet, Copenhagen, Denmark

Local Sympathetic Reflex Mechanism in Regulation of Blood Flow in Human Subcutaneous Adipose Tissue

by

OLE HENRIKSEN

COPENHAGEN 1977

Denne afhandling er i forbindelse med omstående tidligere publicerede afhandlinger af det lægevidenskabelige fakultet ved Københavns Universitet antaget til offentlig at forsvares for den medicinske doktorgrad

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III Henriksen O Effect of somaesthetic denervation upon the vasoconstrictor response in human subcutaneous tissue *Acta physiol scand* 1976. *96* 431–432.

IV Henriksen, O Effect of chronic sympathetic denervation upon blood flow in human subcutaneous tissue. *Acta physiol. scand* 1976 *97* 377–384

V Henriksen, O Local reflex in microcirculation in human subcutaneous tissue *Acta physiol. scand* 1976 *97* 447–456.

VI Henriksen, O Local nervous mechanism in regulation of blood flow in human subcutaneous tissue *Acta physiol scand* 1976. *97* 385–391

Preface

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Ole Henriksen
Rødovre 1977

Introduction

Adjustment of vascular resistance to changes in arterial pressure (autoregulation) and/or changes in venous pressure plays an important role in regulation of blood flow and transcapillary fluid exchange

Autoregulation of blood flow

Autoregulation of blood flow *i e* tendency towards maintenance of constant blood flow during changes in arterial perfusion pressure head has been demonstrated in various tissues and organs in animals brain (Fog 1934 Rapela and Green 1964) kidneys (Rein 1931 Selkurt 1946) skeletal muscle (Folkow 1949 Stainsby and Renkin 1961 Jones and Berne 1964) myocardium (Berne 1959) intestine (Bürgi 1944 Johnson 1960) liver (Torrance 1958) and adipose tissue (Henriksen Nielsen, and Paaske 1976)

Human studies indicate that autoregulation is present in brain (Lassen 1959 Paulson Olesen, and Stig Christensen 1972) and cutaneous tissue (Henriksen *et al* 1973)

The mechanism responsible for adjustment of vascular resistance has been a matter of much controversy The mechanism appears to be very sensitive to local surgical trauma and to changes in local carbon dioxide tension (Häggendahl and Johansson 1965 Paulson Olesen and Stig Christensen 1972) Autoregulation was not abolished by acute and chronic sympathectomy in cat skeletal muscle (Folkow 1949 Jones and Berne 1964) and vasomotoric activity was still present in reserpinized cats (Folkow and Öberg 1961) This indicates that local nervous mechanisms are not involved.

As originally proposed by Bayliss (1902) intrinsic myogenic smooth muscle reactions *related to stretch might be involved (Folkow 1962 and 1964) Another possible explanation* is that vascular resistance adjustments are due to changes in local concentration of vasoactive metabolites liberated from the tissues (Anrep 1912, Berne 1964 Hilton 1971) or variation of local oxygen tension (Ross *et al* 1962) Other theories have been proposed but the fundamental nature of the mechanism is still unsettled (Johnson 1964).

Venous pressure elevation

Venous pressure elevation of 20 mm Hg caused a pronounced increase in vascular resistance in adipose tissue of rabbits (Henriksen, Nielsen and Paaske 1976) Similar findings have been obtained in rabbit ear (Burton and Rosenberg 1956 Rosenberg 1956) cat skeletal muscle (Folkow and Öberg 1961 Mellander Odelram and Öberg 1964) dog limb (Haddy and Gilbert 1956) kidney (Haddy 1956) and in mesentery of different animal species (Selkurt and Johnson 1958 Johnson 1959 Richardson and Zweifach 1970) It was concluded that increase in vascular resistance was due to arteriolar constriction (Haddy

and Gilbert 1956 Haddy and Scott 1964 Richardson and Zweifach 1970 Johnson 1968 Baez *et al* 1974)

In humans venous stasis of 40 mm Hg caused decrease of blood flow in skeletal muscle and cutaneous tissue (Henriksen and Sejrsen 1976 and 1977 a)

Parallel elevation of arterial and venous pressures

Parallel increase in arterial and venous pressures was accompanied by decrease in blood flow in cat skeletal muscle (Folkow and Öberg 1961)

In *humans* parallel increase in arterial and venous transmural pressures can be produced either by lowering of a limb or by applying external negative pressure Limb blood flow measured by venous occlusion plethysmography decreased when vascular transmural pressure was elevated (Gaskell and Burton 1963 Greenfield and Patterson 1954 a, Beaconsfield and Ginsburg 1955 Coles and Greenfield 1956 Mellander Oderam and Öberg 1964) The phenomenon has been demonstrated in human skeletal muscle (Golenhofen and Hildebrandt 1962 Balldin *et al* 1971 Amery *et al.* 1973) and human cutaneous tissue (Henriksen *et al* 1973)

Mechanism

Arteriolar constriction in response to vascular distension might be due to *central reflex mechanisms* Passive tilting of a subject (feet down) caused decrease in blood flow in forearm remaining at heart level The decrease did not occur following sympathectomy (Brigden *et al* 1950) Roddie and Shepherd (1956) and Roddie *et al* (1958) concluded that blood flow in skeletal muscle only was affected by baroreceptor activity Delius *et al* (1972) observed that changes in baroreceptor activity did not influence electrical activity in sympathetic fibres of skin fascicles Also carotid artery occlusion did not affect blood flow in subcutaneous adipose tissue (Ngai *et al* 1966 Hanley Sachs, and Skinner 1971) However DiSalvo *et al* (1971) found that blood flow in subcutaneous tissue was influenced. Similarly other investigators concluded based on indirect evidence, that skin vessels were affected (Mosley 1969 Beiser *et al* 1970 Zoller *et al* 1972 Rowell *et al* 1973)

In animals it has been shown that the local vasoconstrictor response to venous stasis was not affected by central blockade (Rosenberg 1956 Haddy and Scott 1964) indicating that *local mechanisms* are responsible. Haddy (1956) found that the response in kidney was abolished in the denervated organ which suggests that the response might be due to a *local nervous mechanism* as originally proposed by Gaskell and Burton (1953) However Johnson (1959) found in dog mesentery that vascular response to venous stasis was unaffected by procaine and phenoxybenzamine and concluded that the response was due to an *intrinsic myogenic mechanism* (Bayliss 1902) In humans, Gaskell and Burton (1953) and Beaconsfield and Ginsburg (1955) observed that the effect of postural changes upon limb blood flow was unaffected by chronic sympathetic denervation in patients suffering from occlusive arterial diseases as measured by venous occlusion plethysmography

Results obtained in animal studies indicate that vascular response to increase in vascular transmural pressure is due to local mechanisms. Otherwise, the results are conflicting. Venous occlusion plethysmography studies of limb blood flow in humans indicated that nervous mechanisms are not involved in vascular reaction to increase in vascular transmural pressure. However the results may not be conclusive because venous occlusion plethysmography does not give reliable estimates of blood flow when veins are distended (Greenfield and Patterson 1954 b) Venous capacity decreases markedly when venous pressure exceeds 10 mm Hg (Wood and Eckstein 1958) The error may be influenced by the magnitude of blood flow because when blood flow is high the duration of the "initial slope" of the curves becomes very short due to fast filling of veins The problem is further complicated by the fact that perfusion through shunt vessels is included when the region in which blood flow is determined comprises hand or foot.

The mechanism responsible for the vascular response to increase in vascular transmural pressure is still unsettled.

The local isotope washout technique (Kety 1949 Lassen Lindbjerg, and Munck 1964) makes it possible to estimate relative blood flow in a single tissue during venous distension. In the present study ^{133}Xenon was used as tracer which was introduced atraumatically into subcutaneous adipose tissue (Sejrsen 1969 Nielsen 1972 b)

The purpose of the present study was to investigate effects of changes in vascular transmural pressure upon blood flow in human subcutaneous adipose tissue in an attempt to clarify underlying mechanisms.

Methods

Measurement of blood flow in subcutaneous adipose tissue

Theoretical considerations When washout of tracer from a homogeneous tissue with homogenous perfusion is entirely perfusion limited (i e diffusion equilibrium is achieved between tissue and effluent blood) alteration in mean concentration of tracer in tissue with time C(t) can be expressed as

$$C(t)=C(0)\ \exp-((f/\lambda)t) \quad (1)$$

C(0) is concentration in tissue at time zero f perfusion coefficient in ml/g·min and λ tissue to blood partition coefficient in ml/g. Assumptions are zero concentration of tracer in inflowing blood and tracer must leave tissue exclusively by effluent blood. Rearrangement of the equation to express f per 100 g of tissue gives

$$f=k\ \lambda\ 100\ (ml/100\ g\ min) \quad (2)$$

(Kety 1949 and 1951) where k is fractional washout rate constant in min As shown by Sejrsen (1971) [133]Xenon washout from inguinal fat pad in cats follows a monoexponential course. Nielsen (1972 a and b) demonstrated that the [133]Xenon washout method gives a reliable estimate of blood flow in adipose tissue when λ is known. λ is very sensitive to variations in lipid concentration of tissue. However relative blood flow can be determined without knowledge of λ, if λ remains constant. Intravascular blood volume in adipose tissue was found by Paaske and Nielsen (1976) to be about 3 per cent (0 03 ml/g) If vascular volume increases with a factor of 2 during venous distension of 40 mm Hg this implies a change in λ of less than 2 per cent.

A detailed discussion of the [133]Xenon washout method has been given previously by Sejrsen (1971)

Sources of error Due to decrease in linear velocity of the blood during venous stasis [133]Xenon might be redistributed to adipose tissue around veins proximally to the site of application. However when the detectors were collimated to record only from the distal half of the labelled area a decrease in calculated blood flow of same magnitude was obtained indicating that this error was insignificant.

In experiments where lidocaine or phentolamine is injected directly into subcutaneous tissue vascular reactions might be influenced by injection trauma. Nielsen (1972 a) showed that blood flow in adipose tissue returned to normal resting values between 10 and 30 minutes after subcutaneous injection of [133]Xenon in saline. The measurements of the present study were therefore not started until 30 minutes after the subcutaneous injections. Control experiments were performed with subcutaneous injection of isotonic saline Results obtained were almost similar to those obtained from non-traumatized areas. This

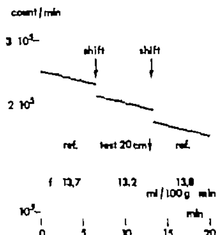


Fig. 1 Example of ¹³³Xenon washout curves from distal part of forearm placed 1) at reference level (jugular notch), f_{ref_1}, 2) lowered 20 cm, f_{test}, and 3) placed at reference level, f_{ref_2}. f denotes perfusion coefficient (ml/100 g min).

indicates that trauma due to infiltration procedures was insignificant at the time of measurement.

Procedure ¹³³Xenon in isotonic saline (3 mCi/ml) was injected intracutaneously into an area located on distal part of forearm or crus About 90 minutes after injection remaining activity is located exclusively in subcutaneous adipose tissue (Sejrsen 1971) At this time measurements were started. The limb under study was carefully immobilized in order to avoid changes in counting geometry due to movements γ-emission of ¹³³Xenon was detected by a scintillation detector (NaI(Tl)) The detector was placed about 15 cm above the radioactive field and collimated to see more than the labelled area. Pulses were fed into a gamma-spectrometer with a window set around the 81 keV photopeak of ¹³³Xenon. Activity was recorded in intervals of 10 or 20 seconds, and results were punched out. ¹³³Xenon washout rate constant k (Eq 2) was computed from the regression line calculated from the "least square" method, using logarithmically transformed count rates corrected for background activity In calculations a λ value of 10 ml/g was used (Sejrsen 1971)

A single investigation consisted of 3 periods of measurement, and each blood flow determination had a duration of about 6 minutes. The sequence of the measurements was 1) reference f_{ref_1}, 2) test, f_{test}, and 3) reference f_{ref_2} (see Fig. 1) Relative blood flow was calculated as $f_{test}/\bar{f}_{ref}$, where f_{test} denotes mean perfusion coefficient (ml/100 g min) obtained under test conditions $\bar{f}_{ref}$ denotes calculated mean value of two measurements obtained under reference conditions just before and after the test.

Changes in vascular transmural pressure were induced by three different techniques, 1) postural changes of a limb 2) local subatmospheric pressure, or 3) venous stasis induced by inflating a cuff placed on upper arm or thigh.

Venous pressure

Venous pressure was measured directly in a superficial vein on the dorsum of the hand in a sitting subject. When the hand was elevated from 5 cm below jugular notch, venous pressure remained constant at about 3 mm Hg When the hand was lowered venous

pressure increased by an amount equivalent to the pressure of a blood column of similar height. During induced local subatmospheric pressure, venous transmural pressure was assumed to increase corresponding to induced negative pressure. During venous stasis venous pressure was found to become close to cuff pressure.

Arterial pressure

Systolic and diastolic arterial pressures were measured on upper arm by means of a cuff. Arterial mean pressure was calculated as diastolic pressure plus 1/3 of the pulse amplitude. During postural changes of the limb, pressure in arterial system of the limb and thus the transmural pressure, was assumed to vary corresponding to the hydrostatic pressure exerted by the column of blood. During application of local suction transmural arterial pressure was assumed to increase corresponding to applied negative pressure. During venous stasis arterial pressure was assumed to remain constant.

Relative vascular resistance

Relative vascular resistance $R_{test}/\bar{R}_{ref}$, was calculated from obtained relative blood flow $f_{test}/\bar{f}_{ref}$, and estimated arterial and venous pressures. R_{test} denotes vascular resistance during test conditions and $\bar{R}_{ref}$ signifies average vascular resistance calculated for the two reference periods.

Statistics

f_{test} and $\bar{f}_{ref}$ were compared by means of Student's t-test for paired samples. Relative blood flow $f_{test}/\bar{f}_{ref}$, obtained under different conditions were compared by randomization test for unpaired samples. As level of significance was chosen 0.05. In figures and text, SD denotes standard deviation, and SE signifies standard error of the mean.

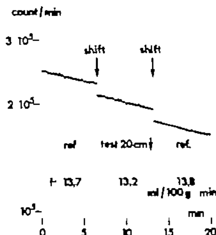


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Results

1 Effect of changes in vascular transmural pressure (I V)

The experiments were performed on 6 healthy subjects Changes in vascular transmural pressure were induced by three different techniques.

(a) Postural changes of a limb

Blood flow was measured either on distal part of forearm with the subject placed in a sitting position or on distal part of crus with the volunteer placed in a supine position Measurements were undertaken at different test levels below or above heart level

Results obtained in one subject are shown in Fig. 2. Blood flow remained constant within 20 cm above and 20 cm below reference level (jugular notch) Raising the arm above this range was followed by decrease in blood flow When the arm was lowered 30 cm below jugular notch, blood flow suddenly decreased 40 per cent of the control value When the arm was further lowered, blood flow remained constant. Calculated vascular resistance at maximum elevation of the arm decreased to 70 per cent of the reference value (venous pressure remained constant) When the arm was lowered to 20 cm below the jugular notch, vascular resistance remained constant (arterial and venous pressures increased in parallel). But when the arm was lowered 30 cm (or more) blood flow suddenly decreased by about 40 per cent. Results were qualitatively similar in all 6 subjects However in one subject blood flow remained constant when the arm was lowered 40 cm (I), and further studies revealed that blood flow decreased in this subject, too when the arm was lowered 50 and 60 cm. Similar results were obtained in the leg.

(b) Local subatmospheric pressure

Effect of local subatmospheric pressure upon blood flow was studied in one subject (I). Blood flow measured on distal part of forearm decreased by about 30 and 50 per cent during negative pressure of −30 and −60 mm Hg, respectively

(c) Venous stasis

Three subjects were studied and blood flow was measured on the distal part of crus During venous stasis of 15 mm Hg blood flow remained constant During venous stasis of 30 and 40 mm Hg, blood flow decreased by about 50 per cent corresponding to an increase in calculated vascular resistance of about 30 per cent

Comments

During elevation corresponding to decrease in arterial mean pressure of about 70 mm Hg blood flow remained constant due to a decrease in vascular resistance. Thus, autoregulation of blood flow is present in human subcutaneous adipose tissue

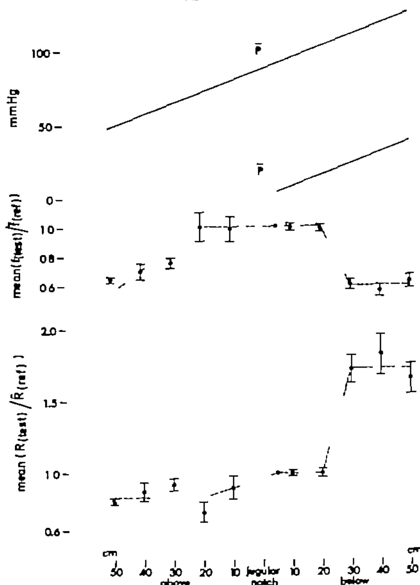


Fig. 2 Mean arterial pressure ($\bar{P}_a$), mean venous pressure, ($\bar{P}_v$), relative blood flow ($f_{test}/\bar{f}_{ref}$) and relative total vascular resistance ($R_{test}/\bar{R}_{ref}$) obtained from distal part of forearm placed at different test levels below or above reference level (jugular notch). Vertical lines with bars denote 1 SD.

When the arm was lowered 20 cm corresponding to an increase in vascular transmural pressure of 15 mm Hg, blood flow and vascular resistance remained constant. This indicates that vascular smooth muscl cells of resistance vessels had increased their active force of contraction just sufficiently to balance the increase in passive distending forces corresponding to the rise in vascular transmural pressure.

When vascular transmural pressure was elevated about 25 mm Hg blood flow decreased due to increase in vascular resistance This response was seen during lowering of the limb during induced local subatmospheric pressure and during venous stasis. When the tissue was infiltrated by histamine which presumably paralyses smooth vascular musculature venous stasis of 40 mm Hg which reduces arteriovenous pressure difference by some 45 per cent. Also it produces a decrease in blood flow of only 18 per cent instead of 50 per cent. This indicates that the decrease in blood flow observed in normal subcutaneous adipose tissue is only partly produced by the drop in driving pressure. To a considerable extent the decrease is caused by an increase in vasoconstrictor activity An increase in total vascular resistance is in agreement with this

Hence moderate change in vascular transmural pressure is followed by regulatory changes in vascular smooth muscle activity keeping blood flow constant (autoregulation) An increase in vascular transmural pressure above a certain *threshold* about 25 mm Hg is followed by an additional vasoconstriction causing a decrease in blood flow of about 50 per cent. This latter phenomenon will for the sake of brevity in the following be referred to as the *vasoconstrictor response*" to an increase in vascular transmural pressure.

Such changes in vascular smooth muscle activity might be due to

1) *Reflex changes* in sympathetic vasoconstrictor activity due to changes in distension of central veins and of the right atrium of the heart (low pressure baroreceptors)
2) *Spinal reflex mechanisms* of local origin *e.g* distension of veins in the extremity might cause arteriolar constriction by way of a reflex arch comprising the spinal cord
3) *Local nervous mechanisms*
4) *Intrinsic vascular adjustments* to changes in vascular transmural pressure (myogenic metabolic)

In order to study the possible role of central mechanisms in the observed changes in vascular resistance during postural changes of a limb experiments were performed before and after an acute spinal sympathetic blockade

2 Acute spinal sympathetic blockade (II)

Both legs of four normal subjects placed in a supine position, were studied. Spinal sympathetic blockade was achieved by epidural anaesthesia induced by marcaine 0.5 per cent, without adrenaline The blockade was extended to involve 6th to 8th thoracic segment. Increase in skin temperature (Roe *et al* 1973) disappearance of sympathicogalvanic reflex (Daos *et al.* 1963) and abolished sweat production as tested by the ninhydrin test (Dhunér *et al* 1960) were taken as evidence of effective central sympathetic blockade.

Arterial blood pressure was measured and remained almost constant throughout the whole investigation.

Plasma concentrations of adrenaline and nor-adrenaline were measured (Valori *et al* 1970) before and after blockade

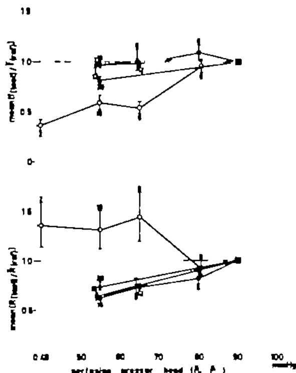


Fig. 8. Mean relative blood flow ±1 SE and total vascular resistance ±1 SE on the distal part of the leg during induced venous stasis plotted as functions of perfusion pressure head. Venous pressure was close to cuff pressure. O = control area infiltrated with isotonic saline. ● areas infiltrated with phentolamine, =areas infiltrated with lidocaine, Δ = areas infiltrated with histamine. Figures denote number of experiments.

A scintigram of the area obtained after conclusion of an experiment showed that activity of ^{133}Xenon remaining in the field was confined to this area. Analgesia of skin was taken as evidence that effective lidocaine blockade had been achieved

(*a*) When the limb was lowered 40 cm, blood flow under control conditions decreased about 60 per cent. This corresponds to increase in calculated vascular resistance of about 200 per cent. In areas infiltrated with lidocaine or phentolamine blood flow remained constant. When the limb was elevated 30 cm blood flow under both control and experimental conditions remained constant (Fig. 7) This corresponds to a decrease in vascular resistance of about 30 per cent.

(*b*) During induced external negative pressure of −30 and −60 mm Hg in one subject, blood flow decreased 30 and 54 per cent respectively in control experiments In areas infiltrated with phentolamine blood flow remained constant.

(*c*) During venous stasis of 15 mm Hg, blood flow remained constant. This corresponds to decrease in vascular resistance of about 15 per cent in control experiments. However under control conditions venous stasis of 30 and 40 mm Hg induced decrease in blood flow of about 50 per cent corresponding to an increase in vascular resistance of about 30 per cent (Fig. 8) Blood flow remained constant in areas infiltrated with lidocaine or phentolamine where decrease in vascular resistance of about 30–40 per cent was found. In order to test effect of low doses of the drugs used, another series of experiments was

XENON WASHOUT CURVES

Fig. 9. ^{133}Xenon washout curves obtained on distal part of forearm. Upper curve shows control experiment with the area infiltrated with isotonic saline. Lower curve shows findings with the area infiltrated with lidocaine ($5 \cdot 10^{-4}$ Mol/l). Venous stasis (30 mm Hg) was induced consecutively 15 25 35 and 45 minutes after injection. Figures above each curve segment denote mean perfusion coefficient ±1 SD. Figures below the curves denote relative blood flow $f_{stasis}/\bar{f}_{ref}$ during venous stasis. $\bar{f}_{ref}$ denotes average value of perfusion coefficients obtained just before and after the stasis period

performed on one subject. Lidocaine was used in concentrations of 3×10^{-4} Mol/l and 5×10^{-5} Mol/l. Control experiments with injection of saline were also performed. Experiments were performed on the distal part of a forearm. Venous stasis of 30 mm Hg was induced consecutively about 15 25 35 and 45 minutes after injections



Fig. 10. ^{133}Xenon washout curves obtained from an area on distal part of forearm infiltrated with phentolamine ($5 \cdot 10^{-4}$ Mol/l). Venous stasis of 30 mm Hg was induced 15 minutes after injection. Figures above the curves denote mean perfusion coefficient ±1 SD. Figure below the curve obtained during venous stasis denotes relative blood flow during venous stasis

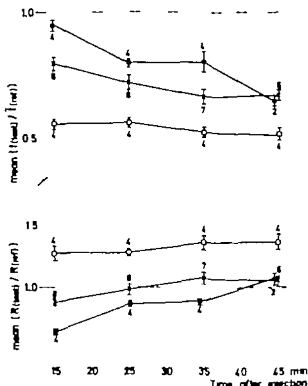


Fig. 11. Mean relative blood flow ±1 SE and mean relative total vascular resistance ±1 SE plotted as functions of time after subcutaneous injection of lidocaine or isotonic saline. Venous stasis of 30 mm Hg was induced 15, 25, 35 and 45 minutes after injection. ● = area infiltrated with lidocaine 3 $\times 10^{-4}$ Mol/L. area infiltrated with lidocaine, 5 $\times 10^{-4}$ Mol/L. ○ = area infiltrated with isotonic saline. Figures denote number of experiments.

An example is shown in Fig. 9. Hyperaemia due to injection trauma was almost identical areas infiltrated with saline and lidocaine. In areas infiltrated with saline blood flow decreased about 90 per cent in all stasis periods corresponding to increase in total vascular resistance of about 30 per cent. Lidocaine (3×10^{-4} Mol/l) blocked response to venous stasis 15 minutes after injection as blood flow remained constant corresponding to decrease in total vascular resistance of about 35 per cent. Lidocaine (5×10^{-4} Mol/l) reduced the response by approximately 50 per cent corresponding to a decrease in vascular resistance of about 15 per cent. However, the effect of lidocaine became less pronounced with time. Similarly phentolamine (5×10^{-4} Mol/l) in two experiments reduced the effect of venous stasis upon blood flow by 50 per cent 15 minutes after the injection (Fig. 10). Results obtained in series with lidocaine and saline are summarized in Fig. 11.

Thus, in presence of lidocaine or phentolamine in very low concentrations venous stasis evoked a *decrease* in total vascular resistance whereas venous stasis evoked an *increase* in vascular resistance during control condition.

RIGHT FOREARM

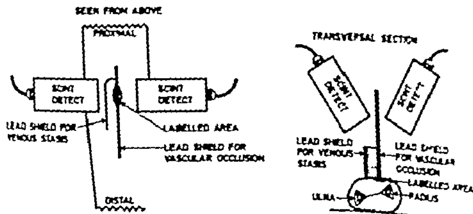


Fig. 12. Experimental "set up". Right: The distal part of the forearm as seen in transversal section. Left: Dorsum of forearm seen from above. A lead shield (14 cm long, 13.5 cm high, and 0.3 cm thick with lower edge narrowed to 1 mm) for vascular occlusion was placed on the skin on dorsal side of forearm. The radioactive field was then separated into two parts. Venous stasis on one side of this shield was induced by another L-shaped lead shield (9 cm long, 5 cm high, and 0.3 cm thick). For details, see text.

Comments

Lidocaine and phentolamine blocked the "vasoconstrictor response" to increase in vascular transmural pressure. This finding speaks against the possibility that sympathetic fibres play a permissive role by exerting a trophic effect on vascular smooth muscle cells. A direct effect of phentolamine and lidocaine on the vascular smooth muscle cells seems to be without significance for the following reasons.

When the areas were elevated 30 cm above mid-axillary line blood flow remained constant corresponding to decrease in vascular resistance of about 30 per cent. This indicates that autoregulation was still present in spite of drugs (Fig. 7). Blood flow and vascular resistance remained constant when the leg was lowered or during local suction (Fig. 7). Thus, vascular smooth muscle cells had increased their force of contraction just enough to counteract the passive distension of the vessels due to increase in transmural pressure. Under these conditions arterial and venous transmural pressures increased in parallel.

Significant difference in response was found between areas infiltrated with lidocaine in low concentrations and control areas infiltrated with saline (randomization test for paired samples). Hyperaemia due to injection trauma was almost identical indicating that difference in response was due to presence of lidocaine. The decrease with time in effect of lidocaine was probably due to washout of drug from the area.

Hence even in very low concentrations lidocaine and phentolamine block the "vasoconstrictor response". This indicates that the response is due to a sympathetic (axon) mechanism. In combination with absence of effect of central sympathetic blockade (III) and somaesthetic denervation (III) these findings indicate that the response is of

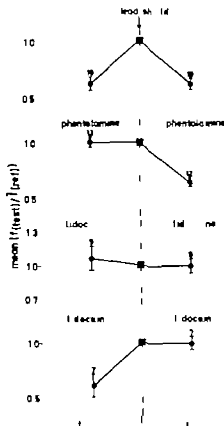


Fig. 13 Mean relative blood flow ±1 SE in human subcutaneous adipose tissue measured simultaneously on both sides of lead shield during venous stasis on one side The figures denote number of investigations.

sympathetic axon reflex mechanism. If the reflex operates in an area of a certain size the vasoconstrictor response should be transmitted from an area with local venous stasis to adjoining areas without stasis

6 Local venous stasis (VI)

Three normal subjects were studied together with two patients who had been bilaterally sympathectomized about two years previously A diagram of experimental "set-up is shown in Fig. 12. [133]Xenon was injected intracutaneously into an area of 3×1 cm which was located longitudinally in the midline on the dorsal side of forearm halfway between elbow and wrist A lead shield (14 cm long, 13.5 cm high and 0.3 cm thick, with the lower edge narrowed to a thickness of 1 mm) was placed longitudinally in the midline on the forearm, thus separating the labelled area into two parts. The shield exerted a pressure on the skin of about 360 mm Hg Venous stasis on one side of the shield was induced by means of a second lead shield (9 cm long 5 cm high and 0 3 cm thick) which was bent into L-shape The second shield was placed proximally and laterally to the labelled area to exert a pressure on the skin of about 40 mm Hg. By this technique venous pressure will rise to

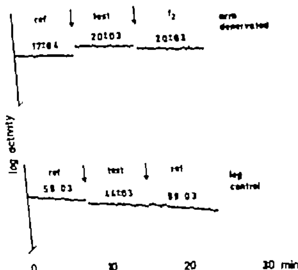


Fig. 14. ^{133}Xenon washout curves obtained on the non-stasis side in a patient who had been bilaterally sympathectomized about 2 years earlier. The curves were obtained during reference conditions, f_{ref}, during venous stasis on opposite side of the lead shield, f_{test}, and finally again during reference conditions, f_{ref}. Measurements were performed on denervated forearm (upper curve) and on non-operated leg used as control (lower curve). Figures denote mean perfusion coefficients ±1 SD (ml/100 g min). Arm: $p>0.6$. Leg: $p<0.01$

about 40 mm Hg in the area between the two lead shields because retrograde venous drainage to veins outside the shields is prevented by venous valves. Blood flow was measured simultaneously on both sides of the lead shield for vascular occlusion.

Results are summarized in Fig. 13. Venous stasis on one side of the shield induced a decrease in blood flow of 40 per cent on both sides. Nerve block was without effect when induced by infiltrating the tissue with lidocaine 3 cm proximally to the labelled area. Response on the non-stasis side could still be demonstrated when venous stasis was induced 1.5 cm in radial direction from the centre of the labelled area. However blood flow was unaffected when the shield for vascular occlusion was placed 3 cm radially to the centre of the labelled area. Blood flow on the non-stasis side was also unaffected when the proximal border of the stasis shield was placed corresponding to the centre of the labelled area.

Phentolamine (10 mg/ml) was injected on the stasis side and blocked the response on this side. The response was still present on the non-stasis side. Lidocaine (20 mg/ml 3×10^{-4} Mol/l) injected on the stasis side blocked the response to venous stasis on both sides (Fig. 13). When lidocaine was injected on the non-stasis side blood flow remained constant on this side but decreased on the side of stasis.

In the sympathetically denervated forearm, blood flow remained constant on the non-stasis side during venous stasis on the opposite side of the lead shield. Blood flow however decreased in the non-operated control leg during venous stasis (Fig. 14).

Comments

Phentolamine or lidocaine injected prior to stasis induced an increase in blood flow on the side of application only. This shows that the longitudinal shield fulfilled its purpose to separate the two sides on microvascular level. Hence induced increase in vascular trans-

mural pressure on one side was not transferred to the other side of the lead shield used for vascular occlusion.

Results indicate that a vasoconstrictor stimulus elicited during venous stasis can be transmitted to a surrounding non-affected area over a distance of about 1.5 cm in the transversal but not in the proximal direction. Nerve block induced 3 cm proximally was without effect, indicating that the "vasoconstrictor response" depends upon a local transmission mechanism

Transmission was unaffected by phentolamine on stasis side whereas by lidocaine and chronic sympathetic denervation transmission was blocked. This indicates that the impulse is transmitted through local nerves

General discussion

Results obtained in the present study indicate that elevation of vascular transmural pressure causes a "vasoconstrictor response" in human subcutaneous tissue. Similar results were obtained during lowering of the limb, during external negative pressure as well as during venous stasis. Same response has also been demonstrated in human cutaneous tissue (Henriksen et al. 1973) and in human skeletal muscle (Golenhofen and Hildebrandt 1964, Bakács et al. 1971, Amery et al. 1973). In animal experiments vasoconstrictor response has been demonstrated in adipose tissue of rabbits (Henriksen, Nielsen and Paaske 1976), in skeletal muscle (Folkow and Öberg 1961), in dog limb (Haddy and Gilbert 1956, Haddy and Scott 1964), in rabbit ear (Burton and Rosenberg 1956), in rabbit limb (Rosenberg 1956) and in mesentery of various species of animals (Selkurt and Johnson 1958, Johnson 1959, Richardson and Zweifach 1970, Baez et al. 1974).

Nature of the "vasoconstrictor response"

The "vasoconstrictor response" in human subcutaneous adipose tissue was elicited when vascular transmural pressure increased by more than some 25 mm Hg (I, V). The response could be transmitted over a distance of about 1.5 cm in transversal direction (VI). The response was abolished by induced local counterpressure (V).

Mechanism

The response was not affected by spinal sympathetic blockade (II), by sympathectomy as measured one day postoperatively (IV), or by local nerve block when induced 3 cm proximally, laterally and medially to the area under study (VI). These findings indicate that local mechanisms are responsible.

A myogenic response (Bayliss 1902, Folkow 1962 and 1964) to increase in vascular transmural pressure might be responsible. Folkow and Öberg (1961) showed that vasomotoric activity was still present in gastrocnemius muscle in reserpinized cats. Passive stretching of a portal vein studied in vitro was followed by an increase in electrical and mechanical activity (Johansson and Mellander 1975). The response was not affected by phenoxybenzamine (5×10^{-4} Mol/l). In vitro studies of small subcutaneous arteries from dogs showed rhythmic activity and coordination, indicating a cell-to-cell transmission of electrical activity (single unit behaviour) (Johansson and Bohr 1966).

Transmission of impulse might be ascribed to longitudinal cell-to-cell transmission of myogenic activity. In vitro studies of turtle arteries and veins by Roddie (1962) indicated that induced electrical activity could be propagated several centimetres. In mammals, peripherally induced vasodilatation was followed by dilatation of femoral artery in dogs

(Hilton 1959) Sympathetic and somaesthetic denervation was without effect. The response was abolished when the artery was divided distally to site of measurement indicating that the dilatator response was conducted longitudinally *via* smooth muscle cells However when repeating the same experiments Mons Lie Sejersted and Kiil (1970) found that dilatator response was still present following transsection of the artery distally to site of measurement. In larger mammalian arteries cell-to-cell propagation of myogenic activity seems to be of minor significance (Burnstock and Prosser 1960 a and b Keatinge 1966) However vascular smooth muscle cells might behave differently in small vessels. Vascular smooth muscle cells showed single unit behaviour and myogenic activity could be transmitted for some distance in rat portal vein (Johansson and Ljung 1967 and 1968 Ljung and Stage 1970 Hermsmeyer 1973) Based on indirect evidence Bevan and Ljung (1974) concluded that myogenic activity could be transmitted over short distances in small arteries of rabbits

Another possibility is that the "vasoconstrictor response" is due to a local nervous mechanism (Gaskell and Burton 1953) The "vasoconstrictor response and impulse transmission was abolished four days and later after sympathectomy (IV VI) Postoperative sweating on third to fourth day following surgery was probably of same nature as degeneration secretion from cat salivary gland (Coats and Emmelin 1962) and to temporary reversion of ptosis 13–15 hours after removal of the superior ganglion in rats (Lundberg 1969) Contents of nor-adrenaline in cat gastrocnemius muscle declined to very low levels four days after sympathectomy (Sedwall 1964) In rat iris only one per cent of sympathetic nerve terminals appeared normal 29 hours after axotomy (Malmfors and Sachs 1965) Postoperative sweating therefore indicates that sympathetic fibres in forearm had lost their ability to function four days after sympathectomy

The effect of sympathectomy upon the vasoconstrictor response" might be due to the fact that degeneration of sympathetic fibres causes changes of vascular smooth muscle cells and affects muscular reactivity and impulse propagation. However initiation and propagation of rhythmic activity in smooth muscles from small subcutaneous arteries in dogs were not affected (Johansson and Bohr 1966) Also reactivity of rat portal vein to ylcholine was not diminished (Johansson *et al* 1969) by chronic sympathectomy Autoregulation of blood flow was still present following sympathetic denervation (IV) Sympathectomy might change lipid metabolism in adipose tissue to affect vascular smooth muscles. However in human cutaneous tissue and human skeletal muscle (Henriksen and Sejrsen 1976 and 1977 a) similar results have been obtained which are not compatible with this theory

Local blockade with lidocaine blocked "vasoconstrictor response and underlying impulse transmission. The latter was not affected by phentolamine (V VI) Selkurt and Johnson (1958) and Johnson (1959) studied the influence of venous pressure elevation upon blood flow and vascular resistance in isolated perfused small intestine in dogs. Total vascular resistance increased during venous stasis. The response was not affected by acute denervation or intraarterially infused phenoxybenzamine or procaine (Johnson 1959) However it seems that blood flow decreased gradually with time in the control experiment presented, and when venous pressure finally was lowered blood flow only increased

corresponding to the increase in perfusion pressure (Selkurt and Johnson 1958). The gradual increase in vascular resistance might therefore be due to intravascular clotting. In cat mesentery Lutz (1966) found that veno-arteriolar response was diminished but not abolished, by guanethidine.

Folkow and Öberg (1961) found that venous stasis of 10 mm Hg induced a decrease in blood flow in skeletal muscle in reserpinized cats indicating that vascular reactivity is still present in more or less complete catecholamine depleted tissue. The duration of measurement was less than 40 seconds. Jones and Berne (1964) observed a transient decrease of blood flow in the thigh muscle of dogs during venous stasis of 20 mm Hg, lasting about 40 seconds, whereafter blood flow almost returned to the level found before stasis. Lundvall and Mellander (1976) made similar observations in skeletal muscle of cats during external negative pressure of −40 mm Hg. Contrary to these findings venous stasis in humans caused a steady decrease in blood flow indicating that the phenomena observed in cat skeletal muscle and the "vasoconstrictor response" to venous stasis in humans are not directly comparable.

Thus the findings in reserpinized cats (Folkow and Öberg 1961) do not exclude an overlying nervous reinforcement mechanism.

Local anaesthetics block conduction of the nervous impulse (Taylor 1959) and phentolamine blocks adrenergic stimulation of "alpha-receptors" in vascular walls (Ahlquist 1948, Roberts *et al.* 1957, Walker *et al.* 1950). The observed effects of local application of these drugs indicate that the "vasoconstrictor response" is due to a local nervous mechanism involving sympathetic adrenergic fibres. However, the effect of phentolamine and lidocaine might be due to direct action on vascular smooth muscle cells (Djojosugito *et al.* 1968) to suppress myogenic activity.

As discussed previously, the drugs did not hamper the capability of vascular smooth muscle cells to produce autoregulatory response. Lidocaine (3×10^{-4} Mol/l) did not affect myogenic activity in rat portal vein, but blocked impulse transmission in sympathetic fibres effectively (Johansson and Ljung 1967). In this concentration, lidocaine blocked the "vasoconstrictor response" 15 minutes after injection. Even when lidocaine (or phentolamine) were applied in concentrations of 5×10^{-4} Mol/l the response was reduced significantly by about 55 per cent.

Furthermore, actual concentrations of drugs in tissue were probably smaller due to dilution. Transmission of the vasoconstrictor stimulus was also blocked by lidocaine (3×10^{-4} Mol/l).

Hence, the effect of lidocaine is due to blockade of impulse transmission in sympathetic fibres, and the effect of phentolamine is due to blockade of alpha-receptors in vascular wall. This indicates that the "vasoconstrictor response" to an increase in vascular transmural pressure is due to a local nervous mechanism involving sympathetic adrenergic fibres.

Site of the reflex

The "*effector site*" of the reflex is probably located in arterioles. Pressure measurements in microvascular system of human cutaneous tissue during changes in orthostatic pressure

and in cat mesentery indicate that precapillary resistance increases during venous stasis (Landis 1929 Richardson and Zweifach 1970) Similarly precapillary resistance increased whereas postcapillary resistance decreased in autoperfused dog forelimb during venous stasis (Haddy and Scott 1964) In rat mesentery Baez *et al* (1974) observed that arteriolar diameter decreased whereas venolar diameter increased during venous stasis.

The "*receptor site*" is probably located in small veins Evidence is obtained from comparison of arterial and venous pressures under various conditions. Thus the response was produced by venous stasis of 30 mm Hg and by lowering the area 40 cm Both procedures produce increase of 30 mm Hg in venous transmural pressure However whereas similar increase in arterial transmural pressure is produced by lowering the extremity increase of maximally 15 mm Hg will occur during venous stasis of 30 mm Hg (Landis 1929) but this increase has already been produced by 20 cm lowering. In this position, blood flow remained constant (I) which contradicts precapillary location of receptor site When increase in venous transmural pressure during 40 cm lowering of the limb was prevented by letting the subject tip his foot, blood flow in subcutaneous adipose tissue remained constant (Henriksen and Sejrsen 1977 b) In this situation, total vascular resistance increased only by 48 per cent corresponding to increase in arterial perfusion pressure head (autoregulation) This is contrary to the increase of about 200 per cent observed in the lowered resting limb Abolishment of the "vasoconstrictor response" during exercise might be due to metabolic changes in subcutaneous adipose tissue. However blood flow at reference conditions was not changed significantly by exercise. Furthermore, when venous pressure was elevated during exercise in the lowered limb by inflating a thigh cuff to 40 mm Hg, blood flow decreased by 50 per cent. This corresponds to an increase in vascular resistance of same magnitude as in the lowered resting leg. In patients with venous insufficiency exercise did not prevent the increase in venous transmural pressure and did not abolish the vasoconstrictor response

Therefore it is concluded that elevation of venous transmural pressure is the factor that elicits the vasoconstrictor response and that the response depends on impulse transmission from veins to arterioles (veno-arteriolar reflex)

The receptors are probably *stretch receptors*" because the reflex was abolished when -us distension was counteracted by inducing local counterpressure (V)

Katz (1950) found that mechanical stretch of free nerve terminals of frog muscle spindles produced a local depolarization in the terminals. When stretch exceeded a certain level, discharge of action potential was *elicited in stem axon. This is in agreement with the* observation that the vasoconstrictor response was not elicited until the venous wall was distended to a degree which corresponds to an increase in venous transmural pressure of about 25 mm Hg. There is however so far no evidence of presence of a similar phenomenon in sympathetic fibres.

Nature of the impulse transmission

The vasoconstrictor response" was not affected by somaesthetic denervation (III) indicating that the impulse is transmitted only in sympathetic fibres. One possibility is that

impulses are transmitted from veins to arterioles in peripheral branches of efferent sympathetic fibres (sympathetic axon reflex) If so it is necessary to assume that a single efferent sympathetic axon innervates both veins and arterioles Lumbar sympathetic nerve stimulation produced an increase in vascular resistance in perfused hind paw of dogs (Zimmerman 1966). The response in arteries and small vessels was blocked by section of somatic nerve bundles accompanying main arterial supply to the paw The response in greater veins was unaffected This indicates that sympathetic fibres supplying arteries, arterioles, and small veins follow the same route. At present, there is no anatomical evidence that a single axon supplies both arterioles and veins.

Another possibility is an axo-axonal (ephaptic) transmission (Furshpan and Potter 1959) between afferent and efferent sympathetic fibres However this seems to be of little significance in mammals In the author's opinion the mechanism is most likely a sympathetic axon reflex. Later studies indicates that the reflex is present in human cutaneous tissue and human skeletal muscle too (Henriksen and Sejrsen 1976 and 1977 a)

Methodological implications of the reflex

Determination of blood flow by venous occlusion plethysmography

Blood flow measured by venous occlusion plethysmography might be influenced by the reflex. When a cuff placed on upper arm was inflated to 40 mm Hg between 40 and 60 seconds were necessary for venous pressure of distal forearm to increase to the threshold level of the reflex (25 mm Hg) (Henriksen and Sejrsen 1977 a) This indicates that *existence of the reflex* does not interfere with measurements of blood flow by venous *occlusion* plethysmography as long as preocclusion venous pressure is kept low

Determination of capillary filtration capacity

Mellander Odelram, and Öberg (1964) determined capillary filtration capacity in human limbs by venous occlusion plethysmography The subject was placed in a supine position and enous pressure was levated 20-30 mm Hg above control values. The vasoconstrictor response which increases pre- to postcapillary resistance ratio was probably elicited in these experiments Estimated capillary pressure was *therefore* too high which leads to underestimation of capillary filtration capacity (0.0077 ml/100 g min mm Hg) Possible involvement of the veno-arteriolar reflex may also be taken into consideration when isogravimetric technique (Pappenheimer and Soto-Rivera 1948) *is used* Using transient osmotic technique (Pappenheimer Renkin, and Borrero 1951) the vasoconstrictor response is not elicited.

Haemodynamic implications of the reflex

Autoregulation of blood flow

Blood flow remained constant during elevation of the forearm *despite decrease* in arterial perfusion pressure head (venous pressure constant). This indicates that autoregulation of blood flow is subcutaneous adipose tissue. *During* increase in vascular

transmural pressure autoregulation was also observed but when venous transmural pressure exceeded a certain magnitude the veno-arteriolar reflex was superimposed. However when the reflex was blocked, autoregulation became evident in the whole range of changes in transmural pressure during lowering the limb as well as during external negative pressure. Similar findings have been obtained in adipose tissue of rabbits (Henriksen Nielsen and Paaske 1976) Ballard and Alhapoulus (1975) found no evidence of autoregulation during arterial hypotension in adipose tissue of dogs. Difference in results obtained might be due to the fact that changes in carbon dioxide tension in tissue, surgical trauma, and mechanical manipulation might affect delicate mechanisms underlying autoregulatory responses (Johnson 1964 Rapela and Green 1964)

Autoregulation was present in denervated tissue (IV) and local nerve block was without effect (V) These findings indicate that intrinsic mechanisms are responsible This is compatible with results in skeletal muscle (Folkow 1949) The exact mechanism cannot be deduced from the present study

Autoregulation might be caused either by myogenic smooth muscle reactions that are related to stretch (Bayliss 1902, Folkow 1962 and 1964 Johnson 1968 Johansson and Mellander 1975) or due to changes in metabolic environment in the tissue (Anrep 1912, Hilton 1971) or both. Stainsby and Renkin (1961) observed that blood flow in skeletal muscle was autoregulated at different levels that correlated to metabolic rate. Furthermore potassium (Kjellmer 1965) and hyperosmolality (Mellander *et al* 1967) seem to play a role in hyperaemia during exercise Cobbold *et al* (1963) showed that effect of sympathetic stimulation was abolished during ischaemia. In human skeletal muscle (Lindbjerg 1969) human cutaneous tissue (Paaske and Henriksen 1975) and human subcutaneous adipose tissue (Nielsen and Sejrsen 1972) "cumulative" blood flow during postischaemic hyperaemia (repayment) was correlated to duration of ischaemia. Fairchild Ross and Guyton (1966) observed that there was no recovery from reactive hyperaemia in the absence of oxygen. It seems more and more evident that oxygen tension influences arteriolar diameter (Duling and Berne 1970 Duling 1972 Hutchins, Bond, and Green 1974) Studies by Gentry and Johnson (1972) indicate that blood flow through capillaries is ned by the condition of arteriolar smooth musculature.

Thus, a basal tonus can probably be ascribed to myogenic reactivity related to stretch whereas autoregulation of blood flow is coupled to metabolic rate *via* metabolites and oxygen tension affecting arteriolar smooth muscle cells. However when venous transmural pressure is elevated about 25 mm Hg a veno-arteriolar reflex is superimposed to cause decrease in blood flow of about 50 per cent. It is important to emphasize that blood flow is still autoregulated at this lower level (I) Oxygen uptake in tissues probably remains constant As determined in blood from femoral artery and vein in humans arteriovenous oxygen difference increased from 43 ml/l to 120 ml/l during passive tilting to 70° and during standing with one leg hanging passively (Reeves *et al* 1961) If oxygen uptake in tissue remains constant this will correspond to a mean decrease in blood flow of about 65 per cent. This would be compatible with findings in subcutaneous adipose tissue of leg (V) and in skeletal muscle (Baldin *et al* 1971 Amery *et al* 1973)

Nicoll and Webb (1955) studied subcutaneous vessels and found that terminal arterioles

were seemingly unaffected by sympathetic innervation. However small arterioles were most sensitive to changes in oxygen tension (Duling 1972 Hutchins Bond and Green 1974) The veno-arteriolar reflex might affect greater arterioles which are richly innervated by adrenergic sympathetic fibres (Ehinger Falck, and Sporrong 1966) whereas intrinsic vascular reactions preferably take place in the smallest arterioles

Trans-capillary fluid transfer

Vascular responses to changes in vascular transmural pressure seem closely connected with regulation of capillary hydrostatic pressure Gore *et al* (1974) found that capillary pressure remained almost constant during arterial hypotension in cat mesentery Järhult and Mellander (1974) studied net transcapillary fluid movements in response to variations in arterial perfusion pressure in acutely sympathectomized cat skeletal muscle Results indicate that within a considerable range capillary hydrostatic pressure remained nearly constant due to resetting of pre to postcapillary resistance ratio. In the lowest pressure range postcapillary resistance suddenly increased. Hanson and Johnson (1962) made similar observations in mesentery of cats However increase in postcapillary resistance was abolished by local infusion of phenoxybenzamine phentolamine or in chronically sympathectomized animals. The increase was present after acute sympathectomy which indicates that this response, too might be due to a sympathetic axon reflex Thus local nervous mechanisms (veno-arteriolar arterio-venous reflexes) seem to participate in regu-lation of capillary hydrostatic pressure to supplement intrinsic vascular reactions. Local nervous reflexes counteract increased fluid filtration induced by augmented vascular transmural pressure under postural changes and venous stasis as edema protecting mechanisms

Cardio-vascular adjustments to postural changes

Changing from supine to upright position caused an average decrease in cardiac output of about 1 7 l/min in resting human subjects (Hanson Tabakin, and Levy 1968) Average body weight was 77 kg. Assuming that mean arterial pressure remains constant at heart level, this decrease in cardiac output corresponds to a reduction in total vascular con-ductance of about 22 per cent. Venous transmural pressure probably increases more than 25 mm Hg in tissues constituting 30 per cent of body weight. Assuming that average decrease in blood flow is 2 ml/100 g min in these tissues the local veno-arteriolar reflex would account for a decrease in cardiac output of approximately 0 77 l/min. This cor responds to a decrease in vascular conductance of about 10 per cent, which is about 45 per cent of total change. The remaining 55 per cent are probably due to central reflex mechanisms elicited from baroreceptors located in arteries central veins and heart (Rod-die and Shepherd 1956 and 1957 Beiser *et al* 1970 Rowell, Wyss and Brengelmann 1973 Di Salvo *et al* 1971) Increase in plasma renin activity (Oparil *et al* 1970) and liberation of adrenaline and nor-adrenaline from adrenal medulla (Celander 1954) might also contribute Hence 45 per cent of increase in vascular resistance due to postural compensation can be ascribed to the veno-arteriolar reflex mechanism that occurs independently of central nervous system

Control of vascular tonus in human subcutaneous adipose tissue

Evidence obtained indicates that vascular tonus is influenced by intrinsic myogenic activity related to stretch by vasoactive metabolites liberated in tissue, and to periarteriolar oxygen tension. However venous distension corresponding to increase in venous pressure of 25 mm Hg or more elicits a veno-arteriolar reflex mechanism which increases arteriolar tonus to supplement effect of myogenic activity These local mechanisms can then be influenced by changes in centrally elicited activity of sympathetic fibres as well as changes in plasma concentration of nor-adrenaline and adrenaline (Celander 1954 Mellander 1960 Mellander and Johansson 1968)

Pathophysiological implications

Arterial insufficiency of legs

The effect of lowering the leg on blood flow in subcutaneous tissue was studied in patients suffering from arterial insufficiency due to iliacofemoral occlusion or severe stenosis (Henriksen 1974) Both vasoconstrictor response and autoregulation of blood flow were present in patients with intermittent claudication without resting pains However in patients with resting pains blood flow increased at base of toes during lowering of leg whereas blood flow decreased at lateral malleolus and fibular head. In these patients "vasoconstrictor response" and autoregulation of blood flow were present in less affected areas whereas both responses were lacking in ischaemic areas Increase of blood flow in ischaemic areas is probably due to decrease in vascular resistance caused by passive distension of vessels due to increase in vascular transmural pressure Furthermore due to the veno-arteriolar reflex blood flow in the leg is redistributed from less affected areas to ischaemic areas creating an inverse steal syndrom (cf Lassen and Westling 1969) Abolishment of reflex and autoregulatory mechanisms is probably due to accumulation of vasodilating metabolites in ischaemic areas to make arteriolar smooth muscle cells less sensitive to constrictor stimuli. In ischaemic areas, it has been shown that these vessels are less sensitive to the vasoconstrictor effect of angiotensine compared to vessels in less [illegible] areas located proximally on the crus (Henriksen and Wisborg 1975)

Venous insufficiency

In normal subjects blood flow in subcutaneous adipose tissue remained constant when the limb was lowered 40 cm if increase in venous transmural pressure was avoided by letting the subject tip the foot continuously However when venous pressure was elevated to about 40 mm Hg by inducing venous stasis blood flow decreased 50 per cent as in lowered resting leg (Henriksen and Sejrsen 1977b) In two patients with venous insufficiency exercise did not abolish the reflex in lowered position. In such patients—contrary to normals—blood flow in subcutaneous tissue of legs is reduced by 50 per cent during walking. This is probably also the case in cutaneous tissue This might play a role in development of crural ulcers in these patients.

Conclusions

The present study provided a number of observations of physiological and clinical significance

1 Activity in sympathetic vasoconstrictor fibres participates in maintenance of vascular tonus in human subcutaneous adipose tissue
2. Following sympathectomy blood flow in human subcutaneous adipose tissue increases by approximately 100 per cent, but vascular tonus is reestablished about 8 days post operatively
3 Autoregulation of blood flow *i.e.* maintenance of constant blood flow during changes in arterial perfusion pressure head or in arterial and venous transmural pressures is present in human subcutaneous adipose tissue.
4 Autoregulation of blood flow was still present in sympathetically denervated tissue and during acute local nervous blockade indicating that intrinsic mechanisms are responsible.
5 Increase in venous transmural pressure of 25 mm Hg or more elicits arteriolar constriction ("vasoconstrictor response to increase in vascular transmural pressure) which reduces blood flow by approximately 50 per cent.
6 The vasoconstrictor response is in all probability due to a local sympathetic axon reflex mechanism.

Further studies

1 Electrical activity in single sympathetic vasoconstrictor fibres should be recorded in order to evaluate whether venous distension is in fact followed by peripherally elicited action potentials.
2. Furthermore it should be tested whether the vasoconstrictor impulse is transmitted from veins to arterioles via an afferent sympathetic fibre which elays in spinal ganglia and an efferent sympathetic fibre with ephaptic coupling. It will be necessary to make unilateral extirpation of spinal ganglia in animal experiments and then await degeneration of peripheral fibres
3 Relative importance between local regulation mechanisms central influence via centrally elicited nervous reflexes and humoral factors should also be studied.

Clinical aspects

1 Effect on blood flow in subcutaneous tissue of lowering the extremity might give information about vascular reactivity in patients with arterial insufficiency ("dependency test") Based on this, it might be possible to predict the effect of vasoactive

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Summary

Effect of changes in vascular transmural pressure upon blood flow in human subcutaneous adipose tissue was studied in normal subjects. Changes in transmural pressure were obtained either by postural changes of a limb by inducing external negative pressure or by inducing venous stasis. Blood flow in subcutaneous adipose tissue was measured by means of the local ^{133}Xenon washout technique

Blood flow remained constant during elevation of a limb 30 cm above heart level corresponding to a decrease in vascular resistance of about 30 per cent (venous pressure remained constant) This indicates that autoregulation of blood flow i maintenance of constant blood flow during changes in arterial perfusion pressure head is present in human subcutaneous adipose tissue Neither chronic sympathetic denervation nor local nervous blockade influenced the autoregulatory response indicating that local intrinsic mechanisms are responsible.

Blood flow remained constant when vascular transmural pressure was elevated 15 mm Hg by lowering the limb 20 cm or by inducing venous stasis of 15 mm Hg However when vascular transmural pressure was elevated 25 mm Hg or more blood flow suddenly decreased about 50 per cent due to an increase in vascular resistance ("vasoconstrictor response"). Similar results were obtained during lowering of the limb during negative external pressure, as well as during venous stasis The vasoconstrictor stimulus could be transmitted to an adjoining non-affected area over a distance of about 1.5 cm

The vasoconstrictor response was blocked when passive distension of the vessels was counteracted by inducing local counterpressure indicating that the vasoconstrictor response is elicited by distension of vessels Blood flow in contralateral leg remained constant.

The vasoconstrictor response to increase in vascular transmural pressure was not affected by acute spinal sympathetic blockade indicating that local mechanisms are responsible Chronic somaesthetic denervation was without effect too The response was still present one day after sympathectomy However four days or later after surgery both the vasoconstrictor response" and the impulse transmission were abolished. Local alpha-receptor" blockade induced by phentolamine blocked vasoconstrictor response but not impulse transmission, whereas lidocaine blocked both.

These findings indicate that the vasoconstrictor response to increase in vascular transmural pressure in human subcutaneous adipose tissue is due to local nervous mechanism involving sympathetic adrenergic fibres probably sympathetic axon reflex The receptor site is most likely located in small veins whereas the effector site is located in arterioles (veno-arteriolar reflex)

The increase in pre to postcapillary resistance ratio induced by the reflex will counteract incre[illegible]illary hydrostatic pressure and supplement intrinsic my

drugs upon blood flow in subcutaneous and presumably in cutaneous tissues too (Henriksen and Wisborg 1975)

2. The "dependency test" might indicate whether functioning sympathetic vasoconstrictor fibres are present in the area in question.
3. Vessels in the leg which are subjected to local orthostatic hypertension compared to those in the arm show an augmented "vasoconstrictor response" (Paaske and Henriksen 1975) Effect of treatment of arterial hypertension might be tested by measuring vascular reactivity
4. In relation to the above-mentioned aspects, it would be interesting to study effect of *temporary hydrostatic hypotension upon the "vasoconstrictor response"* in vessels of the leg with special reference to the time course.
5. It might also be very interesting to study local regulation of blood flow in subcutaneous tissue in patients with vasospastic disorders and in patients with various skin diseases

Resumé

Effekten af ændringer i blodkarrenes transmurale tryk på gennemblødningen i underhudens fedtvæv er undersøgt i forsøg udført på raske mennesker. Transmurale trykændringer blev fremkaldt på tre forskellige måder: 1) stillingsændringer af en ekstremitet, 2) lokalt eksternt, negativt tryk og 3) venestase. Gennemblødningen i subcutant fedtvæv blev målt ved hjælp af den lokale [133]Xenon udvaskningsteknik.

Gennemblødningen forblev uændret, når en ekstremitet blev eleveret 30 cm, hvilket svarer til et fald i den totale karmodstand på ca. 30 %. Dette fund tyder på, at gennemblødningen i subcutant fedtvæv er autoreguleret, dvs. uændret gennemblødning trods ændringer i det arterielle perfusionstryk (venetrykket forbliver uændret under elevation). Det autoregulatoriske respons kunne stadig påvises efter kronisk sympatisk denervering og under lokal nerveblokade. Dette taler for, at autoregulation af gennemblødningen skyldes ændringer i arteriolernes tonus på grund af lokale ikke-nervøse faktorer. Gennemblødningen blev ikke påvirket af 20 cm's nedsænkning af et måleområde på en ekstremitet eller af 15 mm Hg venestase. Derimod blev gennemblødningen pludselig reduceret med ca. 50 % på grund af en stigning i karmodstanden, når det transmurale tryk blev øget med 25 mm Hg eller mer, såvel under sænkning af ekstremiteten, ved eksternt lokalt undertryk, som ved venestase (vasokonstriktor responset). Et vasokonstriktor stimulus kunne transmitteres til et omgivende ikke påvirket område over en afstand af omkring 1,5 cm. Vasokonstriktor responset bliver sandsynligvis udløst ved passiv distension af karrene, da responset kunne blokeres, når man modvirkede distensionen ved at påføre lokalt modtryk over måleområdet. Gennemblødningen i det modsatte ben, der brugtes som kontrol, forblev uændret.

Akut spinal sympaticusblokade havde ingen effekt, hvilket tyder på, at vasokonstriktor responset skyldes lokale faktorer. Kronisk somatisk denervering havde ligeledes ingen effekt. Responset kunne stadig påvises dagen efter sympatektomi, men hverken vasokonstriktor responset eller impulstransmissionen kunne påvises på fjerde postoperative dag eller senere. Lokal alfa-receptor blokade med phentolamin blokerede vasokonstriktor responset, men ikke impulstransmissionen, mens lokalt indgivet lidocain blokerede begge dele. Disse fund tyder på, at det vasokonstriktoriske respons over for stigning i det vaskulære transmurale tryk på 25 mm Hg eller mere skyldes en lokal nervøs refleksmekanisme i sympatiske fibre, dvs. en sympatisk axonrefleks. Receptorstedet er sandsynligvis placeret i de små vener, mens effektorstedet er placeret i arteriolerne (veno-arteriolær refleks).

Stigningen i forholdet mellem præ- og postkapillær modstand, som induceres af refleksen, vil modvirke en stigning i det kapillære hydrostatiske tryk og vil dermed, sammen med den myogene reaktion, modvirke en stigning i den transkapillære filtrationshastighed (beskyttelse mod ødemdannelse).

En mulig udløsning af refleksen i eksperimenter med venestase til bestemmelse af vævsfiltrationskoefficienter bør overvejes. Da den veno-arteriolære refleks også er blevet demonstreret i cutant væv og tværstribet skeletmuskulatur hos mennesker, synes denne mekanisme kvantitativt at spille en vigtig rolle i de vaskulære reaktioner på stillingsændringer.

Blokering af refleksen viste en demaskering af gennemblødningens autoregulation. Kar-tonus synes således at være påvirket af myogen reaktivitet relateret til distensionen af karrene, vasoaktive metabolitter frigjort i vævene og den periarteriolære oxygentension. Venedistension svarende til en transmural trykstigning på 25 mm Hg eller mere udløser en veno-arteriolær refleks, som øger den arteriolære tonus og reducerer gennemblødningen ca. 50%.

Til disse lokale mekanismer adderes påvirkninger betinget af ændringer i aktiviteten i sympatiske fibre udløst centralt fra eller ændringer i plasmakoncentrationerne af adrenalin eller noradrenalin.

Hos patienter med occlusioner eller svære stenoser af arterierne i benene ledsaget af hvileamerter er både det autoregulatoriske respons og den veno-arteriolære refleks blokeret. Dette skyldes formentlig akkumulation af vasodilaterende metabolitter i de iskæmiske væv.

Den veno-arteriolære refleks kan blokeres, når stigningen i det superficielle venetryk under nedhængning af benet undgås ved at aktivere venepumpen ved muskelarbejde. Hos patienter med venøs insufficiens vil muskel-venepumpen ikke kunne sænke venetrykket under gang. Derfor er gennemblødningen i subcutis og sandsynligvis også i cutis hos disse patienter reduceret med 50% under gang i forhold til gennemblødningen ved horisontal lejring, i modsætningen til det man finder hos normale. Dette kunne være en medvirkende årsag til udviklingen af ulcus cruris sekundært til veneinsufficiens.

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